M O N O G R A P H

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A C O N I T I C A C I D

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ACONITIC ACID

Table of Contents

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Summary

The literature available on aconitic acid is limited and deals chiefly with two aspects of the substance: the role of aconitic acid in the citric acid or "Krebs Cycle", and the possible toxicity of the substance as a component of the diet of ruminants - sheep and cattle - and in some instances, of horses.

The investigations of Krebs and Johnson in the early 1930's which led to the citric acid cycle postulated in 1937 have become a classic in modern biology. Carbohydrates, amino acids and fatty acids during respiration yield acetyl groups which enter the tricarboxylic acid or Krebs cycle, progressing through citrate to cis-aconitate to isocitrate to alpha-ketoglutarate to succurate to fumarate to malate to oxaloacete to citrate, closing the cycle. The primary function of this cycle is the dehydrogenation of acetic acid to form two molecules of CO_2 and four pairs of hydrogen atoms.

It is of some interest to note that it is the trans-form of aconitates that appears in the grasses (and other plants such as Equisitum, or "horsetails") which is the subject of toxicity studies comprising the majority of investigations reported upon in this monograph. Transaconitic acid behaves as a competitive inhibitor of the tricarboxylic cycle, and for this reason when the acid was observed in recent years (1960's) to be present in appreciably quantity in range grasses of pastures where periodic epizootics of a disease called "grass tetany" of sheep and cattle were observed, the transaconitic acid became suspect as the toxic agent producing the tetany.

"Grass tetany" has been used to describe a syndrome in ruminants with a variety of speculations regarding the nature or causes of the disease. Hypomagnesemia, hypocupremia, hyperpotassemia, hyperphosphatemia, bilirubinemia, increased blood urea nitrogen, and dehydration are all symptoms of grass tetany. Among the suggested causes of the disease, possible chelation of blood magnesium and calcium by the transaconitic acid in the grasses has been subjected to a number of investigations. These studies have generally failed to support the role of aconitic acid as the toxic agent responsible for grass tetany.

Equisetum, the common "horsetail", has been considered highly toxic to horses who may feed upon it, sometimes fatally. Rapp (65) reported a number of cases of horses having been poisoned by Equisitum, and notes the lack of success in identifying the toxic agent. Aconitic acid, present in the leaves and tubers of the "horsetail", was considered a possible agent, together with unspecified alkaloids.

Camp et al. (12) tested the toxicity of potassium transaconitate on pregnant ewes, dosed orally with the salt, with from 1 g/kg body weight to 4.5 g/kg daily for a number of weeks. Blood analyses were made of the citric, lactic and pyruvic acids; sodium, potassium and calcium serum concentrations; magnesium and phosohorus; serum protein and blood urea nitrogen. The dose level of 4.5 g/kg proved lethal. A

similar experiment intubated the animals with similar doses of transaconitic acid. The aconitate reduced the average serum magnesium and elevated the inorganic phosphorus levels; the acid decreased the serum potassium values and increased the phosphorus. Both aconitate and aconitic acid, in lethal doses to sheep showed electrocardiograms similar to those characteristic of hyperpotassemia. The authors concluded that transaconitate is neither the most essential or most common etiologic factor in grass tetany.

Kennedy (39), also using sheep fed the animals citrates, cis- and transaconitates and injected them intravenously. The oral doses were 0.1 or 0.2 M, the intravenous doses, 1.0 m M/kg body weight. The tolerance of the sheep for transaconitate was so marked that the author considered the substance an unlikely factor in causing hypomagnesemia in sheep.

An enzyme of the citric acid cycle, aconitase, is found in mammalian tissues including the human prostate gland. Cooper and Imfeld (17) studied pathological cases of benign, obstructive and carcinomatous prostates in patients about to undergo prostatectomy. Citric and aconitic acid determinations compared between benign and carcinomatous prostates showed a significant decrease in citrate synthesis in carcinoma - providing advance evidence of malignancy, before histological or clinical techniques might reveal the situation.

Simola and Kosunen (72) fed rats sodium salts of nineteen aliphatic carbonic acids in equivalent doses corresponding to 60 mg Na/100 g body weight, in an experiment to demonstrate the formation of citric acid. Analyses of urinary citric acid showed the greatest conversion to citric acid of pyruvic acid aldol and glutamic acids, with gluconic acid least productive; aconitic acid was intermediate in the ranking of the nineteen acids tested.

During the years following the announcement of Krebs and Johnson of the citric acid cycle, considerable speculation and testing of the hypothesis ensued. Martius (47) dismissed the idea as having significance in carbohydrate metabolism and saw it as of greater significance in amino acid synthesis, since the product, alpha-ketoglutaric acid, could be enzymatically converted to glutamic acid, "a most important amino acid".

The investigations of Lomba et al. (45) into the causes of hypomagnesemia or grass tetany led to a conclusion, shared by other students of the subject, that organic acids in general, and transaconitic acid in particular, are unlikely candidates in producing the disease. Rabbits were intravenously perfused with a number of organic acids, singly or in combinations, at dose levels varying from 0.5 to 10%, of pyruvic, transaconitic, citric, oxalic and mixtures of fumaric, succinic, malonic and malic acids. Blood plasma determinations of calcium and magnesium showed no appreciable relationship between affected blood levels of calcium and magnesium and the symptoms of convulsions and death of grass tetany.

Guinea pigs and sheep were subjected to various treatments with oral and intraperitoneal doses of sodium-trans-aconitate by Wright and Wolff (78). Blood magnesium levels were determined and it was demonstrated that the aconitate produced no toxic effect or

inhibition of the citric acid cycle. The authors concluded that trans-aconitate does not play a major role in controlling serum magnesium levels.

In vitro studies of the anticoagulant effect of various tricarboxylic acids were performed by Gordon (30) who used venous blood from medical patients with normal clotting times. The time interval between the mixing of 2 ml blood sample with the sodium salt of a polycarboxylic acid and the formation of a firm, adhering clot in the test tube was measured. The tricarboxylates markedly increased the clotting time of whole blood; the dicarboxylates were considerably less effective. A direct correlation between concentration of the isocitrate and cis-aconitate and the delay of clotting was observed. The author suggested that the carboxylates influenced the clotting mechanism through chelation of the calcium ion.

ACONITIC ACID

Chemical Information

I. Nomenclature

A. Common Name

Aconitic Acid

B. Chemical Names

1. Aconitic Acid
2. 1,2,3-Propenetricarboxylic Acid
3. Equisetic Acid
4. Citridic Acid
5. Achilleic Acid

C. Trade Names

None

D. Chemical Abstracts Services Unique Registry Number

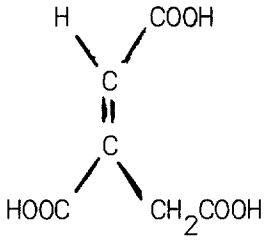
000499-12-7

II. Empirical Formula

$C_6H_6O_6$

III. Structural Formula

$C_3H_3(CO_2H)_3$



(Trans-configuration)

IV. Molecular Weight

174.11

V. Specifications

(GRAS as a synthetic flavoring and adjuvant)

VI. Description

A. General Characteristics

Leaflets or plates, needles from water.

B. Physical Properties

Decomposes 198-199 degrees. Soluble 1 g/5.5 ml H_2O, 13 degrees; 1 g/2 ml 25 degrees. Soluble in 2 parts 88% alcohol, 12 degrees. Slightly soluble ether.

C. Stability

No Information

VII. Analytical Methods

From Poe, W. E.; Barrentine, B. F. (61):

Separation of Aconitic Acid from Sorgo Juice. Approximately 100 ml of juice was centrifuged to separate the starch. The starch-free juice was shaken with Celite filter and and filtered with suction. The clarified juice, which has a pH of 5.0 to 5.5, was lowered to pH 1.3 with sulfuric acid solution to furnish cations to convert aconitates to aconitic acid. Ten milliliters of the juice was shaken 4 minutes with 20 ml of 2-butanone in a centrifuge tube and centrifuged 1 minute to separate the layers.

Color Development. An aliquot of the 2-butanone layer (0.1 or 0.2 ml is usually sufficient for sorgo juices) was evaporated to dryness using a steam bath and a stream of air. Ten milliliters of acetic anhydride and 0.01 ml of pyridine were added. The sample was mixed and after 45 minutes color was read on a Coleman Model 14 spectrophotometer at 550 mu.

Standard Aconitic Acid Curve. Solutions of trans-aconitic acid were prepared in acetic anhydride to produce final concentrations ranging from 2 to 20 ug per ml in a volume of 10 ml. Pyridine (0.01 ml) was added and the solution mixed and read after 45 minutes on the spectrophotometer at 550 mu. Concentration versus absorbance was plotted to give a straight line, accurate between 4 and 20 ug per ml.

VIII. Occurrence

Found in leaves and tubers of Aconitum napellus, members of Ranunculaceae, Achillea and Equisetum. Also found in beets and sugar cane (a commercial source). Manufactured by action of H_2SO_4 or methane sulfonic acid on citric acid.

Biological Data

I. Acute Toxicity

Equisetum, the common "horsetail" plant has been long considered poisonous, especially to horses. Rapp (65) has presented a brief history of the plant's often fatal toxicity for horses. An early description of the symptoms of Equisetum poisoning reads "Loss of coordination of muscular movement, beginning as a slight unsteadiness or uncertainty of gait, most marked when excited" - "The horse finally dies from exhaustion induced by trauma and frequent attempts to rise". Various efforts to identify the toxic principle in Equisetum have been unsuccessful, but one investigator has found aconitic acid in leaves and tubes of Aconitum napellus, and various species of Achillea and Equisetum. The author states that Equisetum poisoning is probably due to aconitic acid and one or more alkaloids.

Camp et al. (12), interested in the toxicity of transaconitic acid relative to grass tetany, determined the LD_{100} of both potassium transaconitate and transaconitic acid to be about 4 g/kg in 10 sheep tested.

II. Short Term Studies

Sheep

It appears that the chief interest in aconitic acid and its salts has been primarily due to its appearance in grasses and other plants used as fodder or grazing by sheep, cattle and horses. Transaconitic acid has been variously suspected as the chief toxic agent in poisoning of animals ingesting it, and as innocent of such claims. Camp, et al. (12), as have other investigators described in this monograph, tested animals on diets including transaconitic acid in an effort to determine its toxicity.

Twelve pregnant ewes were divided into two groups of six each; the control group averaged 39 kg body weight, the treated group, 38 kg. Potassium trans-aconitate, in a 25% solution, was given orally at a dose level of 1.0 g/kg body weight daily for one week; during the following 7-day periods, the dose was increased by 0.5 gm/kg/day to a total of 4.5 gm/kg. Blood samples were taken every 3 or 4 days and analyzed for citric, lactic and pyruvic acids, as well as sodium, potassium and calcium. Magnesium and phosphorus serum concentrations, serum protein and blood urea nitrogen values were determined. The results are shown on the following page:

TABLE 1—Pyruvic, Lactic, and Citric Acid Values in Blood of 6 Control Sheep and of 6 Sheep Given Potassium Transaconitate

Dose of potassium transaconitate given to principals (Gm./kg. of body wt.)	Pyruvic acid (mg./100 ml. ± S.D.)		Lactic acid (mg./100 ml. ± S.D.)		Citric acid* (mg./100 ml. ± S.D.)	
	Controls	Principals	Controls	Principals	Controls	Principals
Before treatment	1.00 ± 0.1	1.10 ± 0.07	15.4 ± 5.2	14.6 ± 12.6	4.97 ± 1.3	3.19 ± 1.2
1.0	1.10 ± 0.1	1.10 ± 0.08	21.5 ± 12.5	17.2 ± 8.6	4.46 ± 1.6	2.34 ± 0.8
1.5	0.91 ± 0.1	0.96 ± 0.06	15.3 ± 6.5	21.3 ± 7.2	4.40 ± 1.8	3.32 ± 1.0
2.0	0.88 ± 0.1	1.23 ± 0.16	9.6 ± 2.8	18.2 ± 10.7	4.23 ± 1.2	3.08 ± 0.9
2.5	0.65 ± 0.1	0.90 ± 0.04	7.6 ± 3.8	18.4 ± 2.8	3.94 ± 1.0	2.85 ± 1.1
3.0	0.71 ± 0.09	0.84 ± 0.09	6.0 ± 2.4	16.9 ± 7.7	3.53 ± 1.5	1.92 ± 0.2
3.5	0.72 ± 0.04	0.86 ± 0.02	6.3 ± 1.8	8.8 ± 1.07	3.92 ± 0.7	1.67 ± 0.3
4.0	0.73 ± 0.01	0.77 ± 0.0	7.8 ± 1.62	9.2 ± 1.7	4.08 ± 0.9	1.63 ± 0.3
4.5	N.D.	0.74 ± 0.0	N.D.	13.3 ± 0.0	N.D.	1.75 ± 0.0

* Assay for citric acid was made on serum.
N.D. = Not determined.

TABLE 2—Electrolyte Values in Serum of 6 Control Sheep and of 6 Sheep Given Potassium Transaconitate

Dose of potassium transaconitate given to principals (Gm./kg. of body wt.)	Sodium (mEq./L. ± S.D.)		Potassium (mEq./L. ± S.D.)		Calcium (mEq./L. ± S.D.)	
	Controls	Principals	Controls	Principals	Controls	Principals
1.0	196.7 ± 35.2	180.0 ± 24.3	5.6 ± 0.3	5.6 ± 0.3	5.2 ± 0.3	4.5 ± 0.4
1.5	160.5 ± 5.3	162.8 ± 5.9	5.4 ± 0.7	5.8 ± 0.5	5.3 ± 0.4	5.4 ± 0.4
2.0	188.7 ± 9.8	191.8 ± 34.4	5.4 ± 0.5	5.6 ± 0.4	5.9 ± 0.3	5.9 ± 0.4
2.5	157.8 ± 12.8	170.0 ± 2.0	5.5 ± 0.2	5.6 ± 0.1	5.4 ± 0.2	6.3 ± 0.2
3.0	156.0 ± 5.5	167.4 ± 8.6	5.2 ± 0.4	5.3 ± 0.1	5.9 ± 0.4	5.8 ± 0.5
3.5	163.2 ± 4.8	174.0 ± 0.0	6.1 ± 0.5	5.4 ± 0.2	5.3 ± 0.4	4.2 ± 0.1
4.0	164.3 ± 7.0	157.0 ± 2.9	6.4 ± 0.3	6.6 ± 0.2	5.8 ± 0.5	4.4 ± 0.5
4.5	N.D.	162.0 ± 0.0	N.D.	6.0 ± 0.0	N.D.	5.0 ± 0.0

N.D. = Not determined.

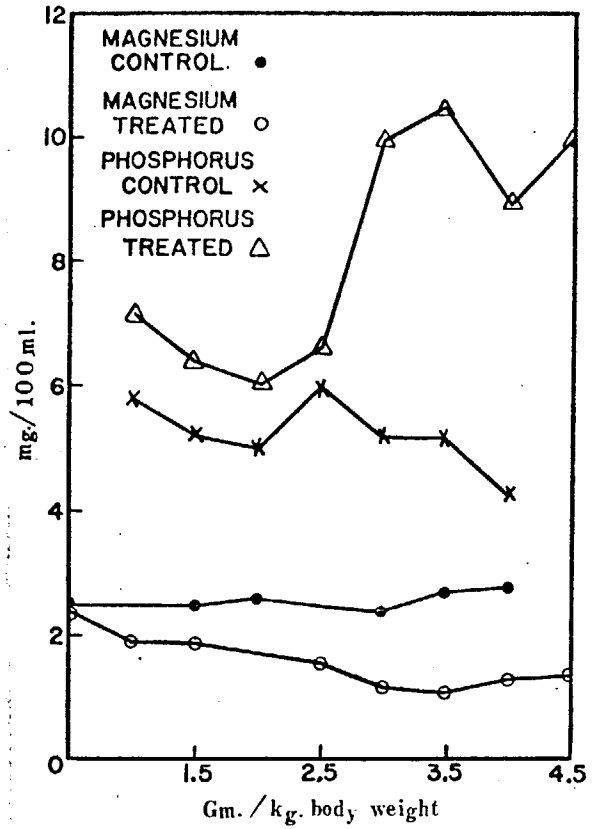


Fig. 1—Serum concentrations (mg./100 ml.) of magnesium and phosphorus in control sheep and sheep given potassium transaconitate (Gm./kg. of body wt.).

TABLE 3—Serum Protein and Blood Urea Nitrogen Values of 6 Control Sheep and of 6 Sheep Treated with Potassium Transaconitate

Dose of potassium transaconitate given to principals (Gm./kg. of body wt.)	Serum protein value (Gm./100 ml. ± S.D.)						Blood urea nitrogen (mg./100 ml. ± S.D.)	
	Globulin		Albumin		Total protein			
	Controls	Principals	Controls	Principals	Controls	Principals	Controls	Principals
1.0	2.7 ± 0.3	3.0 ± 0.5	2.79 ± 0.3	2.60 ± 0.3	5.5 ± 0.3	5.65 ± 0.6	17.7 ± 4.5	10.9 ± 3.9
1.5	2.65 ± 0.4	3.1 ± 0.5	3.28 ± 0.7	2.83 ± 0.3	5.76 ± 0.4	5.82 ± 0.7	17.3 ± 3.3	10.5 ± 5.4
2.0	2.64 ± 0.3	2.73 ± 0.4	3.00 ± 0.2	2.85 ± 0.4	5.63 ± 0.2	5.45 ± 0.6	19.1 ± 4.2	9.7 ± 3.4
2.5	2.23 ± 0.3	2.5 ± 0.5	2.40 ± 0.4	2.40 ± 0.2	4.63 ± 0.1	4.95 ± 0.7	20.1 ± 5.9	6.2 ± 1.5
3.0	2.25 ± 0.2	2.8 ± 0.3	2.60 ± 0.2	2.63 ± 0.2	4.79 ± 0.2	5.46 ± 0.4	19.4 ± 6.5	7.6 ± 1.0
3.5	2.78 ± 0.4	2.88 ± 0.2	2.37 ± 0.2	3.18 ± 0.1	5.13 ± 0.4	6.05 ± 0.0	19.9 ± 4.0	9.4 ± 2.6
4.0	2.90 ± 0.3	2.73 ± 0.0	2.07 ± 0.2	3.10 ± 0.4	4.96 ± 0.2	5.83 ± 0.4	23.3 ± 0.8	8.9 ± 2.4
4.5	N.D.	3.2 ± 0.0	N.D.	3.30 ± 0.0	N.D.	6.5 ± 0.0	N.D.	8.9 ± 2.4

N.D. = Not determined.

In a second experiment, eight sheep of an average weight of 47.75 kg each were divided into two equal groups comprising 2 males and 2 females each. A 25% solution of transaconitic acid was given by stomach tube to the test animals at dose levels of 1.0 gm/kg/day for one week, 1.5 gm/kg/day for 3 weeks, and 2 gm/kg/day for 5 days. Blood samples taken every 3 or 4 days were analyzed for sodium, potassium, calcium, phosphorus, magnesium, citric acid, blood urea nitrogen and serum protein concentrations. The results are shown in the tables on the following page:

In the experiment with the potassium transaconitate, the only significant differences between treated and control animals appeared in the reduction of the average serum magnesium level and the elevation of the inorganic phosphorus values. The transaconitic acid showed a significant decrease in average serum potassium values and an increased phosphorus level. A single lethal dose of 4 gm/kg of both the aconitate and the aconitic acid increased the serum citric acid concentration (from 6.6 to 13.5 mg/100 ml in 24 hours for the potassium transaconitate; from 5.8 to 18.8 mg/100 ml in 8 hours for the transaconitic acid).

Electrocardiograms were made of 5 sheep given a single lethal dose of either the transaconitate (2 of potassium, 1 of sodium) or the transaconitic acid (2 sheep). The ECG was characteristic of that seen in hyperpotassemia.

Necropsies performed within an hour of the death of the animals, and histologic sections of tissues examined, showed nonspecific changes in liver and kidney in the form of lesions characteristic of toxic insult, and which appeared similar to those seen in sheep fed magnesium-deficient diets.

It was concluded that while the transaconitate ion is toxic to sheep, the experimental results did not support the suggestion that the transaconitate ion is either the most common or essential etiologic factor in all forms of grass tetany.

Additional experimental data which excludes transaconitate as a significant cause of the lethality of certain aconitate-concentrations found in forage of ruminants were presented by Kennedy (39) in a study of sheep. It was noted that there was not only a need for additional information concerning transaconitate metabolism, but for data related to the action of the aconitate on rumen microorganisms as well.

Four types of experiments using Merino or Merino X Border Leicester wethers weighing 37-53 kg were performed: in vitro fermentation; intra-ruminal administration of transaconitate and citrate; dietary administration of transaconitate and citrate; and intravenous administration of citrate and cis- and transaconitate.

Three sheep were fed either 0.2 M citrate or 0.2 M transaconitate in the diet for 3 days after which rumen fluid samples were obtained from rumen fistulae and incubated. The fermenting fluid was analyzed spectrophotometrically for aconitate. The citrate, but not the aconitate, was substantially broken down during incubation of the rumen fluid.

Two sheep had rumen fluid removed and mixed with either 0.1 M citrate or transaconitate and then returned to the rumen. The experiment was repeated, with each animal given the alternate treatment.

TABLE 4—Electrolyte Values in Serum of 4 Control Sheep and of 4 Sheep Given Transaconitic Acid

Dose of transaconitic acid given to principals (Gm./kg. of body wt.)	Sodium (mEq./L. ± S.D.)		Potassium* (mEq./L. ± S.D.)		Calcium (mEq./L. ± S.D.)		Phosphorus (mg./100 ml. ± S.D.)		Magnesium (mg./100 ml. ± S.D.)	
	Controls	Principals	Controls	Principals	Controls	Principals	Controls	Principals	Controls	Principals
Before treatment	166.6 ± 24.3	175.3 ± 23.8	6.3 ± 0.5	6.4 ± 0.4	6.5 ± 0.3	7.0 ± 0.3	8.71 ± 2.0	6.86 ± 0.67	1.99 ± 0.3	2.29 ± 0.3
1.0	184.6 ± 19.6	190.3 ± 16.3	6.7 ± 0.4	6.3 ± 0.4	5.7 ± 1.0	6.2 ± 0.6	8.31 ± 1.15	7.21 ± 1.32	1.96 ± 0.3	2.21 ± 0.3
1.5	173.8 ± 19.7	178.6 ± 20.7	5.8 ± 0.9	5.3 ± 1.0	6.0 ± 0.5	6.1 ± 0.4	6.94 ± 0.9	7.25 ± 3.0	2.19 ± 0.0	2.16 ± 0.3
2.0	174.0 ± 10.4	186.0 ± 11.4	5.6 ± 0.4	4.48 ± 0.9	5.7 ± 0.3	5.7 ± 0.1	6.35 ± 0.5	8.83 ± 0.9	2.37 ± 0.4	1.80 ± 0.08

* Data significant at 5% level.

TABLE 5—Citric Acid, Blood Urea Nitrogen, and Serum Protein Concentrations of 4 Control Sheep and of 4 Sheep Fed Transaconitic Acid

Dose of transaconitic acid given to principals (Gm./kg. of body wt.)	Citric acid (mg./100 ml. ± S.D.)		Blood urea nitrogen (mg./100 ml. ± S.D.)		Serum protein value (Gm./100 ml. ± S.D.)					
					Total protein		Albumin		Globulin	
	Controls	Principals	Controls	Principals	Controls	Principals	Controls	Principals	Controls	Principals
Before treatment	5.43 ± 0.9	4.10 ± 1.0	9.5 ± 2.5	10.5 ± 3.7	6.5 ± 0.3	6.9 ± 1.1	3.6 ± 0.5	3.4 ± 0.2	2.9 ± 0.4	3.5 ± 1.1
1.0	5.15 ± 1.4	4.28 ± 1.2	12.5 ± 4.4	8.6 ± 4.2	6.3 ± 0.2	6.9 ± 1.1	3.3 ± 0.5	3.4 ± 0.3	2.9 ± 0.5	3.4 ± 1.1
1.5	5.35 ± 1.4	4.84 ± 1.5	14.8 ± 3.0	14.0 ± 3.9	6.3 ± 0.5	6.5 ± 2.8	3.5 ± 0.3	3.4 ± 0.4	2.8 ± 0.5	3.0 ± 1.0
2.0	5.45 ± 1.0	3.30 ± 0.6	13.2 ± 3.1	23.7 ± 3.0	6.3 ± 0.3	6.6 ± 0.2	3.6 ± 0.3	4.2 ± 0.3	2.7 ± 0.5	2.4 ± 0.3

Samples were again taken and analyzed for volatile fatty acid, polyethylene glycol and organic acids. Both citrate and aconitate disappeared rapidly from the rumen; the citrate increased the volatile fatty acids; the transaconitate did not, although it did slightly increase the blood level of citrate acid aconitate.

The dietary studies involved 10 sheep. Two sheep each were given solutions of transaconitic acid, 0.1 M/day and 0.2 M/day for 5 days; four sheep were similarly treated with citric acid. The remaining two sheep received only the untreated diet. Urine was collected and analyzed for aconitate, as were blood samples. The sheep receiving the transaconitate showed greatly increased urinary citrate levels, but blood citrate and ketone values were essentially the same as those of the controls.

No abnormal behavior was observed in 5 sheep given intravenous transaconitate (C14 in positions 1 and 5) at doses of 1.0 mM/kg body weight. The blood level of the aconitate fell rapidly (only 9% of the injected aconitate could be observed 20 minutes after injection), and rapid renal clearance of the aconitate into the urine was seen. Injection of citrate, also with C14 in positions 1 and 5, at the same levels for the aconitate was highly toxic and killed one animal within 7 minutes. The plasma magnesium and calcium levels remained nearly constant after injection. The tolerance by the sheep for transaconitate was so marked that the author considered it "highly unlikely" that transaconitate could by itself comprise a major factor in causing hypomagnesaemia or lethal metabolic inhibition in sheep.

III. Long Term Studies

None

IV. Special Studies

The role of aconitic acid in the citric acid, or Kreb's, cycle was discussed by Cooper and Imfeld (17) as related to benign and malignant prostatic disease in a group of patients undergoing various types of prostatectomy. One of the enzymes of the citric acid cycle is aconitase, found in mammalian tissues, and particularly for the purposes of this study, in the prostate. Aconitase is concerned with the interconversion of citrate, isocitrate and cisaconitate. The authors studied 77 pathological cases of benign, obstructive prostates and 5 carcinomatous glands; determinations of citric acid and aconitic acid concentrations in the prostatic tissue were made and expressed in milligrams/gm of prostatic tissue. In the 77 benign obstructive prostates, the citrate averaged 2.08 mg/kg and the aconitate averaged 1.14 mg/gm; in the 5 carcinomatious prostates, the citrate average was 0.448 mg/gm and the aconitate, 0.163 mg/gm. Additional observations by the authors suggested that citrate synthesis in prostatic carcinoma is significantly altered and may antedate histological or clinical evidence of malignancy.

ACONITIC ACID

Biochemical Aspects

I. Breakdown

None

II. Absorption - Distribution

None

III. Metabolism and Excretion

The metabolic fate of a series of "aliphatic carbonic acids" and the relationship of these substances to the formation of citric acid was tested by Simola and Kosunen (72) using full-grown rats. Orally administered sodium salts of various acids in equivalent doses were fed to rats; the urine was collected for a 24 hour period and analyzed for citric acid content. The doses corresponded to 60 mg sodium/100 g body weight of the animals; each substance was usually given to two rats. The acids studied and the resultant urinary citric acid excreted, in milligrams and milligram percent of total urine, are shown in the following table:

	A. Verabreichte Substanz	B. Ausgeschiedene Citronensäure pro 24 St. in mg	C. Citronensäuregehalt des Sammelharnes in mg %
1.	Ohne jede Verabreichung	0,8	16
2.	Natriumcarbonat	2,9	44
3.	Brenztraubensäure	11,6	290
4.	Brenztraubensäurealdol	57,0	950
5.	α-Ketoglutarsäure	14,6	366
6.	Bernsteinsäure	35,5	418
7.	Fumarsäure	34,4	275
8.	Maleinsäure	13,7	210
9.	Äpfelsäure	17,3	287
10.	Oxalessigsäure	8,6	216
11.	Normalbuttersäure	15,8	158
12.	Isobuttersäure	9,8	109
13.	β-Oxybuttersäure	14,6	162
14.	Crotonsäure	18,6	133
15.	Malonsäure	37,3	583
16.	Glutarsäure	51 8	1150
17.	Adipinsäure	5,0	200
18.	Citraconsäure	31,9	638
19.	Aconitsäure	32,3	413
20.	Glykolsäure	15,0	158
21.	Gluconsäure	7,9	71

Key:
A.= administered substance B.= citric acid excreted in 24 h in mg.
C.= citric acid content of the total urine in mg%.

1.= without any administration
2.= sodium carbonate
3.= pyruvic acid 4.= pyruvic acid aldol 5.= -ketoglutaric acid
6.= succinic acid 7.= fumaric acid 8.= maleic acid
9.= malic acid 10.= oxalacetic acid 11.= butyric acid
12.= isobutyric acid 13.= β-hydroxybutyric acid
14.= crotonic acid 15.= malonic acid 16.= glutaric acid
17.= adipic acid 18.= citraconic acid 19.= aconitic acid
20.= glycolic acid 21.= gluconic acid

The acids listed in the order from maximum citric acid formation, or excretion, to minimum effect are: pyruvic acid adol, glutaric acid, malonic acid, succinic acid, fumaric acid, aconitic acid, citraconic acid, crotonic acid, malic acid, butyric acid, glycolic acid, alpha-ketoglutaric acid, beta-hydroxybutyric acid, maleic acid, pyruvic acid, isobutyric acid, oxalacetic acid, gluconic acid and adipic acid.

The order of sequence of the acids is somewhat different when viewed as the mg % of total urine, but in both instances, pyruvic acid aldol and glutaric acid produce the greatest citric acid urinary content, and gluconic acid, the least. Additional studies were contemplated to meet objections raised by this experiment.

IV. Effects on Enzymes and Other Biochemical Parameters

Martius (47) discussed in an early paper the relationship of citric acid ⟷ aconitic acid ⟷ isocitric acid as having significance only in the synthesis of amino acids, and he dismissed the concept of Krebs in which the citric acid cycle explained the catabolism of all carbohydrates. Martins noted that the catabolic product of isocitric acid, alpha-ketoglutaric acid, which may be enzymatically converted to glutamic acid, was a "most important amino acid".

Since the years of the postulation of the tricarboxylic acid cycle by Krebs and Johnson in 1937 and publication of Martius' paper in 1939, the "Krebs cycle" has become a classic in modern biology and has been greatly expanded in detail in its relationship to respiration.

The drop in blood magnesium, or hypomagnesemia which characterizes many ruminants feeding on the grasses of early Spring, has been the subject of numerous investigations. The mechanism underlying the changes in blood magnesium chemistry leading to the disturbances or deaths from "grass tetany" in cattle and sheep has been variously attributed to changes in the physiology of the rumen, increased digestive elimination of endogenic magnesium, chelation of the blood magnesium by substances capable of entering the blood from the rumen, etc. The observation that spring grasses in areas of known cases of grass tetany show elevated amounts of transaconitic acid has produced what appears to be in this monograph a major interest in aconitic acid as an agent in the diet of ruminants, responsible for hypomagnesemia or grass tetany.

Lomba et al. (45) approached the problem by suggesting a number of experiments, the first of which they reported here. Rabbits, not identified, were intravenously perfused with various organic acids or mixtures of acids and the blood calcium and magnesium levels determined. The acid solutions were perfused at the rate of 0.5 ml/minute. The concentrations of the acids, neutralized with sodium to a pH of 7.3, were pyruvic acid 2.5 and 10%, transaconitic acid 1.5 and 10%, citric acid 2 and 5%, oxalic acid 0.5 and 2%, and two mixtures, ones of fumaric, succinic, malonic and oxalic acids, and a second of transaconitic, malic and citric acids, all in concentrations similar to that found in grasses.

Blood samples taken before and after perfusion were centrifuged and the plasma calcium and magnesium determined spectrophotometrically. Oxalic acid caused the blood calcium to nearly disappear; citric and transaconitic acids increased the calcemia. Citric and oxalic acids increased the magnesium, the latter only slightly; transaconitic and pyruvic acids produced no effect or change in blood magnesium.

Except for oxalic acid there was no demonstrable relationships between the blood levels of calcium and magnesium and the convulsions and death characteristic of hypomagnesemia.

Toxicities of the perfused acids were also studied, with results varying with dose, speed of injection and interval before death. A comparison of the toxicities puts oxalic acid in first place, citric acid about a third as toxic, and pyruvic acid one-eleventh; transaconitic acid proved by far the least toxic, scarcely one-thirtieth as toxic as oxalic acid.

The authors concluded that the organic acids studied were unlikely candidates in the determination of hypomagnesemial tetany.

The presence of high concentrations of transaconitic acid in range grasses has been suggested as the cause of hypomagnesaemia in cattle and sheep. In vitro experiments have shown that transaconitic acid also inhibits the enzyme aconitase of the Kreb's cycle. Wright and Wolff (78) tested the effects of oral doses of sodium transaconite on the serum magnesium levels of guinea pigs and sheep.

Two groups of seven male guinea pigs, 360-490 g weight, were fed a diet containing 0.19% M g, and were dosed orally (one group) with 3.4 m M/kg body weight of Na-transaconitate (equal to about 1.3% of the acid in the diet). Blood samples were taken before and after dosing, and the serum magnesium levels determined. After a week, the groups were interchanged and the treatment repeated. The treated animals showed only slightly lower serum Mg levels. A similar treatment, but with 6.8% transaconitate in the diet of three male guinea pigs still showed little effect on serum Mg. Six sheep of 20-28 kg weight were given a diet containing 0.28% Mg; three of the animals received 0.29 M (10% of the diet) sodium transaconitate, and three were given 0.87 M $NaHCO_3$ for equivalent sodium ions and served as controls. Blood samples showed no differences in serum Mg at any sampling period.

Intraperitoneal injections in guinea pigs of C^{14}-labelled citric acid or glucose, and trans-aconitate showed no toxic effects of the aconitate and no inhibition of citric acid cycle oxidations. It appeared from all experiments, that transaconitate acid does not play a major role in controlling serum Mg levels.

The similarity of various tricarboxylic acids to citric acid, whose anticoagulant action had been known for some 75 years, led Gordon (30) to test a number of polycarboxylic acids for effect in interfering with the clotting mechanism in human blood. The experiments were performed in vitro, using 2 ml of venous blood from patients having no known clotting defects; each sample was incubated in tubes

with 0.5 ml of the test substance. Controls were blood samples incubated with 0.5 ml of 0.85% saline. Clotting time was defined as the interval between the mixing of blood sample and a sodium salt of a polycarboxylic acid and the formation of a firm clot which clung to the bottom of the tube when inverted. The results are shown in the table below:

TABLE I. Effect of Polycarboxylic Acids on Clotting Time of Human Blood. 2 ml of blood mixed with 0.5 ml of 0.23 M acid (neutralized to pH 7). Control tubes contained 2 ml of blood plus 0.5 ml of 0.85% saline. Values are the means of a number (in parentheses) of experiments. Range of values is given beneath mean value in each case.

Acid	Clotting time	
	Control	Sample
Tricarboxylic	Min.	
Citric* (4)	5	>24 hr
Cis-aconitic (4)	5	>24 "
Trans-aconitic (4)	7	5–18 "
Isocitric (4)	6	>24 "
Isocitric lactone (4)	5	30 min. 13–49 "
Tricarballylic (4)	5	>24 hr
Dicarboxylic	Min.	Min.
α-ketoglutaric (4)	5	10.5 9–12
Succinic (4)	6	28 18–41
Fumaric (4)	6	8 7–9
Malic (7)	5	21 17–42
Glutaric (4)	6	10.5 4–15
Citraconic (3)	7	27 26–29
Glutamic (2)	5.5	10 8–12
Malonic (5)	5	12 8–16

* Concentration .115 M.

Cis-aconitate, trans-aconitate, isocitrate and tricarballylate markedly increased the clotting time of whole blood. In the instances of clotting delay by isocitrate and cis-aconitate, the degree of effectiveness was directly related to the concentration of the substance in the blood. One suggested explanation for the mechanism by which the effective carboxylates operated to delay clotting was in the chelation of Ca++. This explanation was, however, incomplete, and the nature of the interference with the coagulation mechanism was not resolved in these experiments.

V. Drug Interaction

None

VI. Consumer Exposure Information

Beyond the brief note in the Handbook of Food Additives listing "Aconitic Acid (Equisetic Acid, Citridic Acid, Achilleic Acid): GRAS as a synthetic flavoring and adjuvant under Section 121.101", and its description in the Merck Index, citing it as used in manufacture of "itaconic acid used as a plasticizer for buna rubber and plastics", no sources have been found relating to aconitic acid or its salts as a food additive. It is a natural substance, characteristic of plant and animal tissues; its suggested toxicity in the diet of ruminants has not been supported by available literature.

ACONITIC ACID

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Biochemical Changes in Sheep Given Transaconitic Acid

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SUMMARY

Serum concentrations of Na, K, Ca, and Mg, lactic, pyruvic, and citric acids, and proteins were measured in sheep given transaconitic acid or its potassium salt. The most significant changes observed in sheep treated with transaconitic acid were the increase in the organic phosphorus content and the decrease in the magnesium content of serum samples. The syndrome of grass tetany was not seen in the sheep given either the salt or the free acid.

Grass tetany was characterized as hypomagnesemia by Sjollema and Seekles[15] in 1929. Muth and Haag[11] found hypomagnesemia and hypocalcemia in cattle affected with grass tetany. Singer *et al.*[14] described a syndrome in ruminants as complicated grass tetany which was characterized by one or more of the following abnormalities: hypomagnesemia, hypocupremia, hypocalcemia, hyperpotassemia, hyperphosphatemia, bilirubinemia, increased blood urea nitrogen (BUN), and dehydration. In the winter wheat grazing area of the United States, a condition called "wheat pasture poisoning" sometimes develops in cattle allowed to graze on the growing wheat plant. The changes in blood electrolytes of diseased cattle are usually characterized by decreased calcium, magnesium, and inorganic phosphate levels and by normal or increased potassium levels.[5]

Several attempts were made to explain the appearance of grass tetany in ruminants. Initially, the disease was viewed as a metabolic disorder resulting from specific dietary factors, such as high potassium or protein content of spring grass,[16] or from environmental stresses that, through neural and endocrine mechanisms, led to physiologic dysfunction. Head and Rook[7] suggested that there was decreased absorption of magnesium from the intestinal tract that was associated with increased production of ammonia in the rumen. Kemp and 'T Hart[8] concluded that there is a direct relationship between the incidence of grass tetany and the K:Ca+Mg ratio in the ration. In 1961, Burt and Thomas[2] suggested that dietary citrate was a contributory factor of grass tetany in animals ingesting lush grass because of the high citric acid

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TABLE 1—Pyruvic, Lactic, and Citric Acid Values in Blood of 6 Control Sheep and of 6 Sheep Given Potassium Transaconitate

Dose of potassium transaconitate given to principals (Gm./kg. of body wt.)	Pyruvic acid (mg./100 ml. ± S.D.)		Lactic acid (mg./100 ml. ± S.D.)		Citric acid* (mg./100 ml. ± S.D.)	
	Controls	Principals	Controls	Principals	Controls	Principals
Before treatment	1.00 ± 0.1	1.10 ± 0.07	15.4 ± 5.2	14.6 ± 12.6	4.97 ± 1.3	3.19 ± 1.2
1.0	1.10 ± 0.1	1.10 ± 0.08	21.5 ± 12.5	17.2 ± 8.6	4.46 ± 1.6	2.34 ± 0.8
1.5	0.91 ± 0.1	0.96 ± 0.06	15.3 ± 6.5	21.3 ± 7.2	4.40 ± 1.8	3.32 ± 1.0
2.0	0.88 ± 0.1	1.23 ± 0.16	9.6 ± 2.8	18.2 ± 10.7	4.23 ± 1.2	3.08 ± 0.9
2.5	0.65 ± 0.1	0.90 ± 0.04	7.6 ± 3.8	18.4 ± 2.8	3.94 ± 1.0	2.85 ± 1.1
3.0	0.71 ± 0.09	0.84 ± 0.09	6.0 ± 2.4	16.9 ± 7.7	3.53 ± 1.5	1.92 ± 0.2
3.5	0.72 ± 0.04	0.86 ± 0.02	6.3 ± 1.8	8.8 ± 1.07	3.92 ± 0.7	1.67 ± 0.3
4.0	0.73 ± 0.01	0.77 ± 0.0	7.8 ± 1.62	9.2 ± 1.7	4.08 ± 0.9	1.63 ± 0.3
4.5	N.D.	0.74 ± 0.0	N.D.	13.3 ± 0.0	N.D.	1.75 ± 0.0

* Assay for citric acid was made on serum.
N.D. = Not determined.

content of some plants. They proposed that citric acid could interfere with the absorption of magnesium and thus produce magnesium deficiency.

In 1965, Burau and Stout[1] found transaconitic acid (0.1 to 12.2%) in samples of range grass collected from pastures where epizootics of grass tetany had periodically occurred. Transaconitic acid is a competitive inhibitor of the tricarboxylic acid cycle. It inhibits the enzyme aconitase which catalyzes the conversion of citric acid to isocitric acid. Since aconitic acid and citric acid form chelates with calcium and magnesium, Stout *et al.*[17] reasoned that these complexes could be expected to affect the normal transference pathways of calcium and magnesium within the animal.

The present study was undertaken to determine the effects of transaconitic acid on certain biochemical constituents of the blood of sheep.

Materials and Methods

Potassium Transaconitate.—Twelve pregnant ewes were allotted to 2 groups of 6 each. Ewes in the principal group ranged in weight from 30 to 44 kg., with an average weight of 38 kg. each. The control ewes ranged in weight from 29 to 48 kg., with an average weight of 39 kg. each.

Each day, the principal sheep were given an oral dose of potassium transaconitate solution. The 25% solution was prepared by dissolving a commercial grade of transaconitic acid in distilled water and then adjusting the solution to pH 7 with potassium hydroxide. The transaconitate solution was given to the principals at the initial dose level of 1.0 Gm./kg. of body weight per day for 7 days, and then the dose was increased in increments of 0.5 Gm./kg. for each succeeding 7-day period. Samples of blood were collected every 3 or 4 days from each ewe, and analyses were made of individual samples.

Transaconitic Acid.—Eight sheep were allotted to 2 groups, each group consisting of 2 males and 2 females. The principal and the control sheep ranged in weight from 39.0 to 52.0 kg., with an average weight of 47.75 kg. each.

A 25% solution of free acid was administered by stomach tube; the initial dose was 1.0 Gm./kg. per day for 7 days. The dose was increased to 1.5 Gm./kg. per day and given at this level for 21 days. Then the dose was increased to 2.0 Gm./kg. per day and given for 5 days. Samples of blood were collected every 3 or 4 days from each sheep, and analyses were made of individual samples.

Ration.—In both feeding experiments, the sheep were maintained on a ration of Sudan hay, salt, and water which were made available ad libitum.

Chemical Analyses.—Analyses of serum samples for sodium, potassium, and calcium were done with the spectrophotometer* with flame attachment, using the acetylene-oxygen burner. The spectral emission of each electrolyte was measured at the following wave length: sodium, 589.0 mμ; potassium, 770.0 mμ; and calcium, 622.7 mμ.[10] For determinations of sodium and potassium, the serum was diluted (1:50) with distilled water and analytical results were read directly on the flame photometer; for calcium, the serum was diluted (1:25) with a 4.5 %

* Model DU Spectrophotometer, Beckman Instrument Company, Fullerton, Calif.

TABLE 2.—Electrolyte Values in Serum of 6 Control Sheep and of 6 Sheep Given Potassium Transaconitate

Dose of potassium transaconitate given to principals (Gm./kg. of body wt.)	Sodium (mEq./L. ± S.D.)		Potassium (mEq./L. ± S.D.)		Calcium (mEq./L. ± S.D.)	
	Controls	Principals	Controls	Principals	Controls	Principals
1.0	196.7 ± 35.2	180.0 ± 24.3	5.6 ± 0.3	5.6 ± 0.3	5.2 ± 0.3	4.5 ± 0.4
1.5	160.5 ± 5.3	162.8 ± 5.9	5.4 ± 0.7	5.8 ± 0.5	5.3 ± 0.4	5.4 ± 0.4
2.0	188.7 ± 9.8	191.8 ± 34.4	5.4 ± 0.5	5.6 ± 0.4	5.9 ± 0.3	5.9 ± 0.4
2.5	157.8 ± 12.8	170.0 ± 2.0	5.5 ± 0.2	5.6 ± 0.1	5.4 ± 0.2	6.3 ± 0.2
3.0	156.0 ± 5.5	167.4 ± 8.6	5.2 ± 0.4	5.3 ± 0.1	5.9 ± 0.4	5.8 ± 0.5
3.5	163.2 ± 4.8	174.0 ± 0.0	6.1 ± 0.5	5.4 ± 0.2	5.3 ± 0.4	4.2 ± 0.1
4.0	161.3 ± 7.0	157.0 ± 2.9	6.4 ± 0.3	6.6 ± 0.2	5.8 ± 0.5	4.4 ± 0.5
4.5	N.D.	162.0 ± 0.0	N.D.	6.0 ± 0.0	N.D.	5.0 ± 0.0

N.D. = Not determined.

10^{-5} M solution of $LaCl_3$ and then the analytical results were read directly on the flame photometer. Lanthanum was added to remove the interference of the phosphate ion.[12]

Standard procedures were used to assay samples of blood for lactic acid,* pyruvic acid,** citric acid,[3] inorganic phosphorus,[6] magnesium,[13] BUN,[4] total serum protein (TSP),† and individual serum proteins.‡

Electrocardiogram.—Five male sheep were trained to stand quietly in a restraining stanchion. Stainless steel electrodes were placed subcutaneously in the sheep. A direct-writing recorder § recorded standard and augmented limb leads and the bipolar sternal (M-X) lead. A lethal dose (4 Gm./kg.) of either transaconitate (pH 7) or transaconitic acid was administered by stomach tube to each sheep. Two sheep were given the potassium form, and 1 was given the sodium salt of transaconitic acid.

Necropsy.—Necropsies were done on all but 3 sheep. Postmortem examinations were made within 1 hour of death of sheep. Tissues were fixed in 10% formalin buffered to maintain a pH of 7. The histologic sections were stained with hematoxylin and eosin stain. Tissues shown to have pigment were stained in accordance with Perls' Prussian blue technique for ferric ion.

Results

Potassium Transaconitate.—Significant differences in citric, lactic, or pyruvic acid were not observed between principal and control sheep, but a trend toward diminution of the citric acid level was seen in sheep treated with potassium transaconitate. Results of the assays conducted on these constituents are summarized (Table 1).

In the instance of a sheep given a lethal dose (4 Gm./kg.) of potassium transaconitate, the citric acid concentration progressively increased from 6.6 to 13.5 mg./100 ml. in 24 hours.

There were no significant differences in the average serum sodium, potassium, or calcium values between principal and control sheep (Table 2). The reduction in the average serum magnesium content for principal sheep was significant at the 0.001 probability level. In addition, the increase in the inorganic phosphorus values of the treated sheep was significant at the 0.05 level (Fig. 1). Neither the TSP nor the individual serum protein values were significantly altered in the principal sheep (Table 3).

Transaconitic Acid.—There was a significant decrease (0.01 level) in the average serum potassium value for sheep given transaconitic acid. There were no significant differences between principal and control sheep with regard to magnesium and inorganic phosphorus content of the serum (Table 4). There was a trend toward a decrease in the average serum citric acid content of sheep given transaconitic acid (Table 5). Moreover, the inorganic phosphorus level increased from 8.3 to 22.5 mg./100 ml. during the period. Neither the TSP nor the individual

* Technical Bulletin No. 825-UV, Sigma Chemical Company, St. Louis, Mo., 1965.
** Technical Bulletin No. 725, Sigma Chemical Company, St. Louis, Mo., 1965.
† Goldberg Refractometer, American Optical Company, Buffalo, N.Y.
‡ Manual RM-1M-3, Beckman Instrument Company, Fullerton, Calif., 1965.
§ Physiograph, E&M Instrument Co., Houston, Texas.

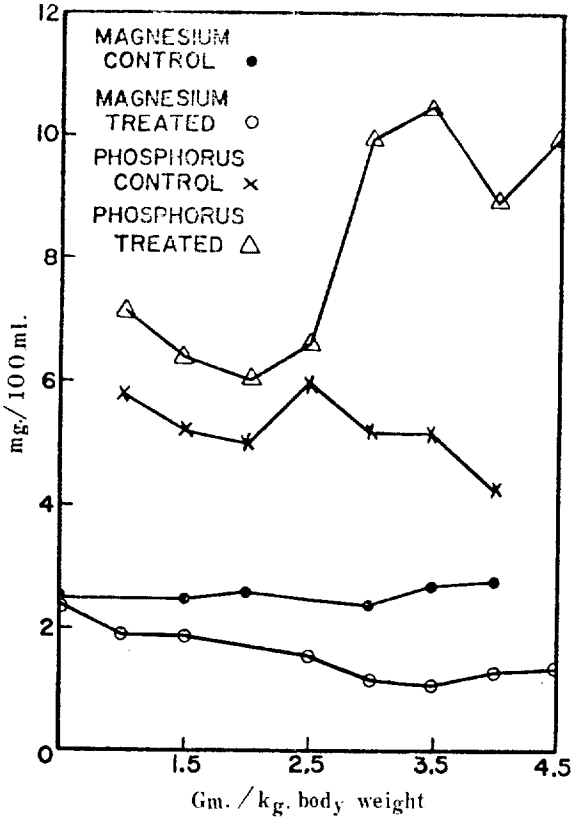

Fig. 1—Serum concentrations (mg./100 ml.) of magnesium and phosphorus in control sheep and sheep given potassium transaconitate (Gm./kg. of body wt.).

serum protein values were significantly changed in principal sheep.

With the sheep given the single lethal dose (4 Gm./kg.) of transaconitic acid, the serum citric acid concentration increased progressively from 5.8 to 18.8 mg./100 ml. in 8 hours.

Electrocardiogram.—The changes in the electrocardiogram (ECG) of the 2 sheep given lethal doses of potassium transaconitate included disappearance of the P wave and then increase in the amplitude of the T wave and depression of the S-T segment. The only change in the ECG of the sheep given sodium transaconitate was fluctuation in heart rate. One of the 2 sheep given a lethal dose of the free acid had an abnormal ECG which was recorded approximately 6 hours after the administration of the free acid when suddenly the heart became ir-

TABLE 3—Serum Protein and Blood Urea Nitrogen Values of 6 Control Sheep and of 6 Sheep Treated with Potassium Transaconitate

Dose of potassium transaconitate given to principals (Gm./kg. of body wt.)	Serum protein value (Gm./100 ml. ± S.D.)						Blood urea nitrogen (mg./100 ml. ± S.D.)	
	Globulin		Albumin		Total protein			
	Controls	Principals	Controls	Principals	Controls	Principals	Controls	Principals
1.0	2.7 ± 0.3	3.0 ± 0.5	2.79 ± 0.3	2.60 ± 0.3	5.5 ± 0.3	5.65 ± 0.6	17.7 ± 4.5	10.9 ± 3.9
1.5	2.65 ± 0.4	3.1 ± 0.5	3.28 ± 0.7	2.83 ± 0.3	5.76 ± 0.4	5.82 ± 0.7	17.3 ± 3.3	10.5 ± 5.4
2.0	2.64 ± 0.3	2.73 ± 0.4	3.00 ± 0.2	2.85 ± 0.4	5.63 ± 0.2	5.45 ± 0.6	19.1 ± 4.2	9.7 ± 3.4
2.5	2.23 ± 0.3	2.5 ± 0.5	2.40 ± 0.4	2.40 ± 0.2	4.63 ± 0.1	4.95 ± 0.7	20.1 ± 5.9	6.2 ± 1.5
3.0	2.25 ± 0.2	2.8 ± 0.3	2.60 ± 0.2	2.63 ± 0.2	4.79 ± 0.2	5.46 ± 0.4	19.4 ± 6.5	7.6 ± 1.0
3.5	2.78 ± 0.4	2.88 ± 0.2	2.37 ± 0.2	3.18 ± 0.1	5.13 ± 0.4	6.05 ± 0.0	19.9 ± 4.0	9.4 ± 2.6
4.0	2.90 ± 0.3	2.73 ± 0.0	2.07 ± 0.2	3.10 ± 0.4	4.96 ± 0.2	5.83 ± 0.4	23.3 ± 0.8	8.9 ± 2.4
4.5	N.D.	3.2 ± 0.0	N.D.	3.30 ± 0.0	N.D.	6.5 ± 0.0	N.D.	8.9 ± 2.4

N.D. = Not determined.

TABLE 4—Electrolyte Values in Serum of 4 Control Sheep and of 4 Sheep Given Transaconitic Acid

Dose of transaconitic acid given to principals (Gm./kg. of body wt.)	Sodium (mEq./L. ± S.D.)		Potassium* (mEq./L. ± S.D.)		Calcium (mEq./L. ± S.D.)		Phosphorus (mg./100 ml. ± S.D.)		Magnesium (mg./100 ml. ± S.D.)	
	Controls	Principals	Controls	Principals	Controls	Principals	Controls	Principals	Controls	Principals
Before treatment	166.6 ± 24.3	175.3 ± 23.8	6.3 ± 0.5	6.4 ± 0.4	6.5 ± 0.3	7.0 ± 0.3	8.71 ± 2.0	6.86 ± 0.67	1.99 ± 0.3	2.29 ± 0.3
1.0	184.6 ± 19.6	190.3 ± 16.3	6.7 ± 0.4	6.3 ± 0.4	5.7 ± 1.0	6.2 ± 0.6	8.31 ± 1.15	7.21 ± 1.32	1.96 ± 0.3	2.21 ± 0.3
1.5	173.8 ± 19.7	178.6 ± 20.7	5.8 ± 0.9	5.3 ± 1.0	6.0 ± 0.5	6.1 ± 0.4	6.94 ± 0.9	7.25 ± 3.0	2.19 ± 0.0	2.16 ± 0.3
2.0	174.0 ± 10.4	186.0 ± 11.4	5.6 ± 0.4	4.48 ± 0.9	5.7 ± 0.3	5.7 ± 0.1	6.35 ± 0.5	8.83 ± 0.9	2.37 ± 0.4	1.84 ± 0.08

* Data significant at 5% level.

TABLE 5—Citric Acid, Blood Urea Nitrogen, and Serum Protein Concentrations of 4 Control Sheep and of 4 Sheep Fed Transaconitic Acid

Dose of transaconitic acid given to principals (Gm./kg. of body wt.)	Citric acid (mg./100 ml. ± S.D.)		Blood urea nitrogen (mg./100 ml. ± S.D.)		Serum protein value (Gm./100 ml. ± S.D.)					
					Total protein		Albumin		Globulin	
	Controls	Principals	Controls	Principals	Controls	Principals	Controls	Principals	Controls	Principals
Before treatment	5.43 ± 0.9	4.10 ± 1.0	9.5 ± 2.5	10.5 ± 3.7	6.5 ± 0.3	6.9 ± 1.1	3.6 ± 0.5	3.4 ± 0.2	2.9 ± 0.4	3.5 ± 1.1
1.0	5.15 ± 1.4	4.28 ± 1.2	12.5 ± 4.4	8.6 ± 4.2	6.3 ± 0.2	6.9 ± 1.1	3.3 ± 0.5	3.4 ± 0.3	2.9 ± 0.5	3.4 ± 1.1
1.5	5.35 ± 1.4	4.84 ± 1.5	14.8 ± 3.0	14.0 ± 3.9	6.3 ± 0.5	6.5 ± 2.8	3.5 ± 0.3	3.4 ± 0.4	2.8 ± 0.5	3.0 ± 1.0
2.0	5.45 ± 1.0	3.30 ± 0.6	13.2 ± 3.1	23.7 ± 3.0	6.3 ± 0.3	6.6 ± 0.2	3.6 ± 0.3	4.2 ± 0.3	2.7 ± 0.5	2.4 ± 0.3

regular with the disappearance of the P wave. Complete heart block occurred less than 1 minute after the initial arrhythmia. The sheep died 25 to 30 minutes after the first sign of arrhythmia.

Pathologic Changes.—Three sheep given the potassium salt of transaconitic acid once a day were necropsied. The main pathologic changes included toxic tubular nephritis; mild to severe fatty metamorphosis of the hepatic parenchyma; iron-negative, brown, granular pigment in hepatic cytoplasm; moderate alveolar emphysema; pale and flaccid heart; serous fluid in the pericardial sac; and focal hemorrhages in the thalamus.

Abnormalities in tissues of 4 sheep given free acid once a day included toxic tubular nephritis; pale yellow liver; fatty metamorphosis of the hepatic parenchyma; iron-negative, brown, granular pigment in the hepatic cytoplasm; pyknosis and karyorrhexis with eosinophilic condensation in the row of hepatic cells adjacent to the central veins; pulmonary congestion and alveolar edema; alveolar emphysema; flaccid and pale heart; ecchymosis in the epicardium over the ventricles; petechiae and ecchymoses in the thymus; and petechiae in walls of the duodenum and anterior part of the jejunum.

On necropsy, 1 sheep given the lethal dose of potassium salt of transaconitic acid had only a pale and flaccid heart. The sheep given the lethal dose of the sodium salt of transaconitic acid had pale yellow liver; fatty metamorphosis of the hepatic parenchyma; pyknosis and karyorrhexis in the cells adjacent to the central veins; hepatic cytoplasm with brown, granular pigment which was iron-negative; pulmonary congestion and alveolar edema; ecchymosis of the epicardium over the ventricles; petechiae and ecchymoses in the thymus; and separation of the outer molecular layer of the cerebral cortex.

The sheep given the lethal dose of free acid had toxic tubular nephritis, hemosiderin in the tubular cytoplasm, pulmonary congestion, and alveolar edema.

Discussion

Transaconitic acid is a competitive inhibitor of aconitase which catalyses the conversion of citric acid to cisaconitic and isocitric acid in the Krebs' cycle. Accumulation of citric acid in the body with formation of magnesium and calcium chelates could produce a condition such as grass tetany.

Potassium Transaconitate.—There was progressive decrease in the average concentration of magnesium in sheep given potassium transaconitate, but tetany was not observed. One sheep had serum magnesium concentration which decreased to 0.2 mg./100 ml., but the sheep did not have tetanic convulsions. The absence of signs of tetany in this sheep may be attributed to the normal calcium concentration which was maintained. An increase in the concentration of inorganic phosphorus of the serum is sometimes indicative of renal insufficiency; however, there was not a concomitant increase in the BUN content of the serum associated with the hyperphosphatemia. Singer *et al.*[14] observed hyperphosphatemia in their investigation of grass tetany in ruminants.

One of the most marked findings in the present experiment was the ability of the treated animals to maintain normal serum levels of citric, lactic, and pyruvic acids, whereas the sheep given the lethal dose of potassium transaconitate had a twofold increase in the serum citric acid content within a 24-hour period. This discrepancy between the studies of acute and chronic feeding could be resolved by suggesting that the glucose is shunted via the pentosephosphate scheme as the sheep on the chronic regimen compensates for the inhibition of the Krebs' cycle.

Transaconitic Acid.—Hyperpotassemia is usually associated with acidosis, whereas in the present study, hypopotassemia developed in the sheep treated with free acid. The amount of transaconitic acid entering the tubules of the kidneys may have excluded the excretion of ammonia, and the excess acid carried potassium with it into the urine.

The average citric acid content of the serum did not increase as would be anticipated if the Krebs' cycle were inhibited. However, when the sheep was given the acute lethal dose of the free acid, the citric acid content of the serum increased from 5.8 to 18.8 mg./100 ml. in an 8-hour period. Moreover, there was not a concomitant change in the calcium or magnesium concentration of the serum of this sheep.

Electrocardiogram.—The changes in the ECG of sheep given an acute, lethal dose of either the free acid or the potassium salt were typical of the changes seen with hyperpotassemia.

Pathologic Changes.—The hepatic and renal lesions are interpreted as being related to the toxic insult in the sheep. The changes were nonspecific, but similar lesions have been reported in sheep fed magnesium-deficient rations.[9]

Conclusion

The transaconitate ion is toxic to sheep; however, the mode of action of this substance cannot be attributed to any specific biochemical constituent which was assayed in this investigation. From results of the present study, it is not possible to conclude that transaconitate ion is the most common or essential etiologic factor for all forms of the entire grass tetany complex.

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THE ROLE OF CITRIC ACID IN THE PHYSIOLOGY OF THE PROSTATE: A PRELIMINARY REPORT

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The physiological role of citric acid or its salts in the mammalian prostate is poorly understood. Scheele (1784)[1] first extracted crystalline citric acid from lemon juice. Schersten (1930)[2] identified it as tricarboxylic compound, citric acid, in animal tissue and found it to be a constituent of milk, bone, urine, and semen. He found seminal citrate to originate in mammalian accessory organs of reproduction, in the prostate gland of man, in the seminal vesicles of the bull, boar, and rabbit. It is the intention of the authors to present a brief history of the biological aspects of citric acid, its relation to the citric acid cycle in general, and to the pathological human prostate in particular.

CITRIC ACID CYCLE

Martius (1937),[3, 4] Krebs and Johanson (1937),[5] and Krebs (1940)[6] evolved the concept of the citric acid cycle, or Kreb's cycle, a common biochemical mechanism for the oxidation of carbohydrates, amino acids, and fatty acids, within the cell. Such metabolic fuels are capable of being chemically reduced to an intermediate member of the citric acid cycle and thus completely oxidized to carbon dioxide and water. The major physiological function of the citric acid cycle in mammalian tissue is the production of energy. It probably represents the main source of energy for all the functional activities of the cell, including growth and multiplication.

Several of the individual stages that constitute the concatenation of the citric acid cycle were demonstrated as early as 1911 by Batelli and Stern[7] when they noted the rapid oxidation of citrate, succinate, fumarate, and malate in frog muscle preparations. The last major step of the cycle was proposed by Krebs and Johnson (1937) when the formation of citrate from oxaloacetate and pyruvate was demonstrated. A simplified representation of the citric acid cycle is given in figure 1.

The citric acid cycle was originally proposed as a mechanism for the terminal oxidation of carbohydrate, but subsequent studies made it obvious that the citric acid cycle is also operative in the oxidation of particular amino acids, fatty acids, and ketone bodies. The chief pathway in the oxidation of such substances in animal tissues involves a series of chemical reactions by which the substrate is prepared for entry into some intermediate stage of the citric acid cycle. The final stage in which ultimate oxidation and release of energy are achieved develops through the successive steps of the citric acid cycle. Among the amino acids oxidized via the citric acid cycle, glutamic acid is of significance to urology since it enters the cycle as a result of a vigorous transamination reaction. Barron and Huggins (1946)[8] noted large amounts of glutamic acid in human adenoma ranging from 50-200 mg./100 gm. tissue. Prostatic tissue slices manifested vigorous transaminase activity when incubated at 38C with buffered glutamate and oxaloacetate or pyruvate. The resultant chemical reaction produces chiefly α-ketoglutaric acid, an intermediate of the citric acid cycle, plus aspartate and alanine which are easily incorporated in the citric acid cycle via oxaloacetate and pyruvate respectively.

Krebs (1954)[9] favors the view that the citric acid cycle is operative in animal tissues generally and has pointed out that no major, alternative oxidative pathway has yet come to light. Each stage of the citric acid cycle requires a specific enzyme system. Pure forms of the specific enzymes are difficult to obtain. Only condensing enzyme and fumarase have been secured in crystalline form. The former is necessary for the formation of citrate from oxaloacetate and coenzyme A. Many and varied studies have been

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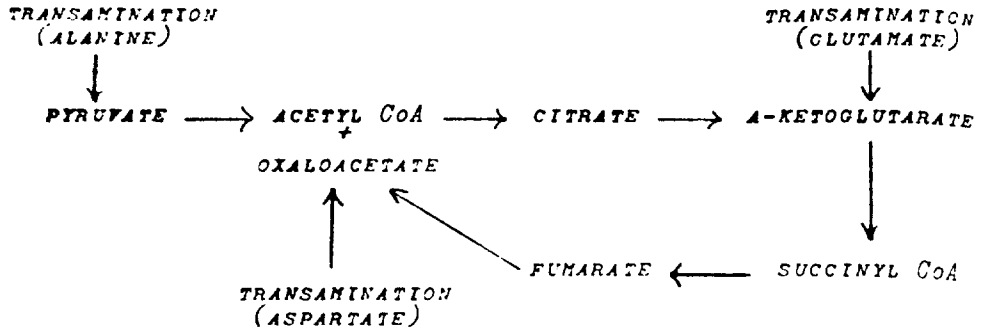

FIG. 1. Simplified schema of citric acid cycle

carried out on biological tissues and fluid in the determination of the various intermediates of the citric acid cycle. More significant than the fact that the intermediates of the citric acid cycle are readily demonstrable in mammalian tissue, is the finding that most of the enzymes responsible for the sequence of the citric acid cycle are also present.

Weinhouse, Millington and Wenner (1950)[10] and Wenner, Spirtes and Weinhouse (1950)[11] reported the presence of the citric acid cycle in malignant tumors and noted the occurrence of three enzymes of the citric acid cycle, condensing enzyme, aconitase, and isocitric dehydrogenase in the tumors studied. Condensing enzyme is of prime importance in the formation of citric acid and the eventual oxidation of citric acid to the next intermediate stage in the citric acid cycle, α-ketoglutaric acid, is potentiated by aconitase and isocitric dehydrogenase.

Barron and Huggins (1946),[8] in an excellent and preliminary paper on the metabolism of the prostate, concluded that it is probable that the citric acid cycle does not exist in prostatic adenoma since they noted that citric acid and α-ketoglutaric acid are not utilized by prostatic tissue slices in vitro. Whether this conclusion is valid is problematical. There is significant biochemical evidence that the citric acid cycle is operative in the prostate in view of the high citrate and aconitase values, as well as the considerable glutamic acid concentration and transaminase activity. In addition there is a major technical criticism that can be made of Barron and Huggins's (1946) study. In adding citric acid and α-ketoglutaric acid to prostatic tissue slices their experimental result is complicated by the intrinsically high prostatic tissue concentrations of such substances. Thus their simple observation that no citrate utilization occurs under such conditions does not imply a lack of citric acid cycle kinetics in the prostate. In addition a further significant fact lies in the observation of Humphrey and Mann (1949)[12] that seminal plasma contains an inhibitor which prevents the oxidation of citrate by animal tissues. In liver slices, citrate utilization can effectively be inhibited by this agent. It may well be that the lack of utilization of citrate in Barron and Huggins's (1946) study is due to the seminal inhibitor described by Humphrey and Mann (1949).

SERUM CITRATE VALUES

In addition to participation in the Krebs cycle, citric acid has several other functions, chiefly involving the humoral, genitourinary, and osseous systems. Primarily the normal range for serum citrate is 1.5-2.7 mg./100 cc. Canary and Kyle (1957)[13] in a study of the serum citrate values in 50 normal patients found a mean value of 2.0 mg. ± 0.5 mg. per 100 cc. Hypercitricemia has been reported in association with the hypercalcemia of hyperparathyroidism, vitamin D toxicity, osteolytic metastases, and prolonged immobilization. Hypocitricemia has been noted in hypoparathyroidism and vitamin D deficiency. Intravenous infusion of calcium and/or saline can increase serum citric acid from 0.6-3.7 mg.

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per cent. Carlsson and Hollunger (1953)[14] noted a marked increase in serum citric acid following an infusion of vitamin D. In two significant papers Harrison (1953)[15] and Kissin and Kreeger (1954)[16] demonstrated hypercitricemia in Paget's disease. In a series of 15 patients with Paget's disease, Kissin and Kreeger found 9 or 60 per cent had abnormal serum citric acid levels. The highest value for the Paget's group was 4.0 mg. per cent. The mean value for Paget's was 2.79 mg. per cent. Control value in 41 patients was 2.0 mg. per cent. The studies cited suggest that the fluctuations in serum citric acid in a variety of skeletal disorders are related to the basic disease process rather than to change in serum calcium.

In a control series of 108 outpatients we found an average serum citrate of 1.924 mg. per cent. The figure compares favorably with the average serum citrate value of 2.0 mg. per cent as reported by Canary and Kyle (1957).[13] The serum citrate values were also serially studied in a group of patients undergoing various types of prostatectomy, for benign and malignant prostatic disease. A total of 22 cases were observed, 18 for benign prostatic hypertrophy and four with carcinoma of the prostate. Two of the malignancies had advanced metastatic disease in bone. Serial determinations of citric acid and aconitic acid were performed immediately after operation, in mid-operation, at the close of the procedure, and 1 hour postoperatively. No elevations of serum citrate or aconitate were observed during the operative procedure. In the men with metastatic prostatic malignancy no elevations of serum citrate or aconitate were found on repeated examinations during the course of the advanced illness.

SEMINAL CITRATE VALUES

Schersten (1929)[2] (1936)[17] noted that seminal fluid rapidly decolorized methylene blue on the addition of "citrico-dehydrogenase" prepared from cucumber seeds. The factor responsible for the decolorization was identified on crystallization as citric acid. In addition Schersten found citric acid present in the seminal plasma of man and proved it to be of prostatic origin.

Harvey (1951)[18] reported 725 semen determinations for citric acid and found a range of 0–2340 mg./100 cc, with a mean value of 479 mg./100 cc. Huggins and Neal (1942)[19] noted a range of 480–2688 mg./100 cc in 9 samples of pure prostatic secretion. In 2 samples of seminal vesicular secretion 15 and 22 mg./100 cc of citric acid were found.

In any consideration of prostatic citric acid production emphasis must be placed on the hormonal relationships that exist in the mammalian species. Humphrey and Mann (1948)[12, 20] and Mann and Parsons (1950)[21] have established the direct relationship between seminal citric acid production and androgens. Castration results in a gradual loss in seminal citrate as well as fructose. Administration of parenteral androgen restores the seminal citrate values to normal, but the restoration is gradual in contrast to the rapid rise in fructose. After several weeks' postcastration, the semen and accessory reproductive organs are completely depleted of citrate and fructose. Hypophysectomy has the same hormonal effect as castration. Humphrey and Mann (1949)[12] have suggested the use of seminal citrate production as a sensitive indicator of androgenic elaboration, since after the administration of testosterone to castrates seminal citrate and fructose can be detected long before significant secretory activity can be observed histologically in the accessory organs.

The prostate is rich in citric acid and aconitase,[22] the enzyme necessary for the chemical interconversion of citrate, cis-aconitate, and isocitrate. It was originally thought that the enzymatic mechanism for the synthesis of citric acid involved the primary formation of aconitic acid, with citric acid being formed secondarily. Stern and Ochoa (1949)[23] found, however, that aqueous extracts of pigeon liver formed citrate initially when acetate, oxaloacetate, coenzyme

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A, condensing enzyme, and Mg or Mn ions were present. Thus the concept of the "condensation reaction" originated a mechanism of prime importance to citrate synthesis in animal tissues. Aconitase is a significant enzyme necessary for the operation of the citric acid cycle, but concerned only with the interconversion of the three substances of the aconitase system, citrate, isocitrate, and cisaconitate. It is necessary for the dehydration of citrate and isocitrate, with cis-aconitate as an intermediate. The interconversion is represented by the schema:

$$\text{citrate} \xrightleftharpoons{Ac} \text{cis-aconitate} \xrightleftharpoons{Ac} \text{isocitrate.}$$

It is now evident that as a result of the condensation reaction citrate is formed primarily and in the presence of aconitase interconverted into cis-aconitate and isocitrate. Aconitase is therefore not necessary for citrate synthesis as conceived by Barron and Huggins (1946).[3]

Huggins and Neal (1942)[19] have suggested that prostatic citrate is concerned with the coagulation and liquefaction of semen. They noted prolonged clotting times of mixtures of blood and seminal fluid. Since citrate has a binding effect on calcium ions, the coagulation defect could well be explained on this basis. Schersten (1936)[17] also postulated the binding action of citrate for calcium in prostatic fluid, but believed that such an affinity prevented the precipitation of calcium salts. Lundquist (1947)[24] has suggested that citric acid may be an activator of prostatic acid phosphatase.

PROSTATIC TISSUE ASSAY IN ADENOMA AND CARCINOMA

In our laboratory we have utilized the method of Saffran and Denstedt (1948)[25] to determine the citric and aconitic acid content of prostatic tissue. The procedure is an application of the Furth-Herrmann reaction,[26] providing a simpler and more rapid method than the laborious pentabromoacetone procedures for citric acid. A total of 77 pathologically proven, benign, obstructive prostates and five carcinomatous glands have been studied by this method. The tissue samples were removed at operation and a weighed portion immediately homogenized in 5 per cent trichloroacetic acid solution in a Potter-Elvehjem apparatus. The citric acid and aconitic acid concentrations were determined colorimetrically in milligrams per gram of prostatic tissue according to Saffran and Denstedt. A summary of the results can be reviewed in table 1. In the benign group of 77 prostates the citric acid values ranged from 0.092–4.02 mg. per gram of tissue, with an average value of 2.08 mg./gm. The aconitic acid values in the group ranged from 0.0–4.60 mg. per gram of tissue, with an average value of 1.14 mg./gm.

The five histologically proven carcinomas of the prostate gave significantly lower values than the benign glands, yielding a range of 0.00–0.882 for citric acid and 0.00–0.382 for aconitic acid. The average citric acid concentration in prostatic cancer was 0.448 mg. gm. and for aconitic acid 0.163 mg./gm. From these figures it is apparent that the synthesis of citrate and its conversion to cis-aconitate may be altered in the malignant prostate.

In view of the fact that ranges and average values may be statistically misleading the total number of prostates studied were reduced to their individual pathological diagnosis and the average citrate and aconitate values recorded. In table 2 can be seen the various benign conditions studied with the average values recorded. In 77 specimens, 65 cases histologically demonstrated the typical fibromuscular and glandular hyperplasia of benign prostatic hypertrophy. The average citrate and aconitate values in this group were higher than the over-all average figure, being

TABLE 1. *Over-all citrate and aconitate values in benign and malignant prostates*

	mg./gm.
A. Benign obstructive prostates:	
1. Total cases—77	
2. Citrate range	0.092–4.02
Average	2.08
3. Aconitate range	0.00–4.60
Average	1.14
B. Carcinomatous prostates:	
1. Total cases—5	
2. Citrate range	0.00–0.882
Average	0.448
3. Aconitate range	0.00–0.382
Average	0.163

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TABLE 2. *Analysis of benign obstructive prostates*

Pathology	No.	Range		Mean Value	
		CA	AA	CA	AA
		mg./gm.	mg./gm.	mg. gm.	mg. gm.
1. Fibromuscular and glandular hyperplasia	65	0.16–4.54	0.0–4.60	2.31	1.30
2. BN contracture or median bar	8	0.09–1.42	0.0–0.73	0.44	0.22
3. Miscellaneous					
A. Infarction	2	0.43–1.34	0.1–0.19		
B. Estrogen	1	0.84	0.87		
C. Incidental	1	0.56	0.23		

recorded as 2.31 and 1.30 mg. gm., respectively. A bladder neck contracture and or median bar was found in eight specimens pathologically, giving average citrate and aconitate values of 0.442 and 0.222 mg. gm. It is obvious that the absence of secretory glands and the presence of practically pure fibromuscular tissue in these surgical specimens are responsible for the relatively low values. In the miscellaneous group, two specimens showing multiple foci of infarction also gave relatively low concentrations of citrate and aconitate as noted. One case of a deliberately estrogenized individual gave values of 0.845 mg. citric acid/gm. and 0.870 mg. aconitic acid, gm. of tissue. An incidental prostate assayed after prostatocystectomy for vesical cancer gave values intermediate between the estrogenized and infarcted prostatic specimens.

In the accumulation of data an attempt was made to correlate citrate and aconitate values from various lobes of the individual prostate glands removed at operation. Perusal of table 3 reveals five benign prostates and three carcinomatous glands that were studied in this fashion. In all the citrate determinations except two, the figures are fairly comparable, indicating that the citrate concentration may be expected to be uniform throughout the organ. In general, no reliable correlation was evident from the aconitic values, although in one or two instances correlative figures were obtained.

In the surgical specimens studied, 15 histologically benign prostates were found to have citric acid concentrations of less than 1.0 mg./gm. of tissue studied. In view of the suspicion that this group might contain latent, undetected carcinoma, a careful clinical and microscopic review was made. From table 4 it is evident that the low citrate concentrations were present in 4 cases of

TABLE 3. *Correlation of citrate and aconitate values from various lobes of prostate*

			CA	AA
			mg./gm.	mg./gm.
1. H.G.	BPH	RL	1.110	0.400
		LL	1.500	0.570
2. J.S.	BPH	RL	4.260	3.600
		ML	3.040	3.350
		LL	3.500	2.850
3. I.H.	BPH	RL	2.840	0.827
		LL	3.210	0.422
4. H.M.	BPH	RL	1.340	0.910
		LL	0.911	0.150
5. L.B.	BPH	RL	0.580	0.290
		LL	0.529	0.170
1. J.W.	CA	ML	0.000	0.000
		RL	0.000	0.000
		LL	0.000	0.000
2. S.G.	CA	PL	0.732	0.183
		RL	0.515	0.289
		LL	1.400	0.617
3. E.P.	CA	RL	0.727	0.072
		LL	0.815	0.068

bladder neck contracture and/or median bar, 8 cases of adenoma with predominantly fibromuscular hyperplasia, and three miscellaneous cases of estrogenization, infarction, and prostatic tissue from a prostatocystectomy for squamous carcinoma of the urinary bladder. In three of the 8 cases of adenoma with low citrate concentrations biopsy has been repeated perineally with the Silverman needle technique. All three were negative for tumor. The remaining 5 cases in the group have been only recently operated on and show no clinical, biochemical, or histological evidence of malignancy. In view of the fact that the citrate values in this small group approximate the values

noted in histologically proven carcinoma, it is planned to keep them under close surveillance with periodic prostatic biopsies in the future.

AUTOPSY VALUES

In view of the observation that the citrate and aconitate concentrations in prostatic tissue decrease directly with time (table 5), it is probable that the values obtained from autopsy material are not reliable. In a brief review of 12 autopsied males ranging in age from newborn to 67 years a variable series of values was obtained. Three children were examined, two newborns and one 9-year-old boy dying of leukemia. It is interesting that no citric acid or aconitic acid could be detected in the prostatic tissue of these prepubertal subjects, substantiating the androgenic dependence of citrate and aconitate. The values in the 9 adult males ranging from 26-67 years were variable and probably serve only as rough estimations of the intrinsic prostatic values for citrate and aconitate. It is interesting that the average values for the men below 50 years of age were significantly lower than the older group. Below 50 years the average citrate and aconitate values were 0.925 mg./gm. and 0.245 mg./gm. Over 50 years the values were 1.410 mg./gm. and 0.759 mg./gm., respectively. In comparison with the average values taken from healthy adenoma at the operating table, 2.08 mg./gm. for citric acid and 1.14 mg./gm. for aconitic acid, the autopsy values are considerably reduced in magnitude, probably secondary to postmortem, intracellular autolysis. However, they are reproduced here for the record.

TABLE 4. *Benign prostates with citrate below 1.0 mg./gm.*

	CA	AA	Pathology	Rebiopsy
	mg./gm.	*mg./gm.*		
1. 3/7 H.P.	0.79	0.73	BPH, FM+	neg.
2. 3/21 H.A.	0.94	0.45	BPH, FM+	neg.
3. 4/26 A.B.	0.97	0.71	BPH, FM+	neg.
4. 7/2 R.S.	0.14	0.00	BN contracture	
5. 8/26 R.D.	0.45	0.62	BPH, chr. inflamm.	
6. 10/8 E.F.	0.09	0.00	BN contracture	
7. 11/1 I.H.	0.96	0.40	BPH, FM+	
8. 11/5 W.A.	0.43	0.11	multiple infarcts	
9. 12/2 B.G.	0.22	0.00	BPH, FM+	
10. 12/3 G.L.	0.16	0.00	BPH, FM+	
11. 12/5 L.B.	0.56	0.24	incidental	
12. 12/9 S.B.	0.79	0.29	BPH	
13. 12/16 A.J.	0.30	0.00	median bar	
14. 12/17 R.W.	0.14	0.00	median bar	
15. 12/26 A.K.	0.85	0.87	estrogenized	

TABLE 5. *Loss of citrate and aconitate with time*

				CA	AA
				mg./gm.	*mg./gm.*
1. J.M.	70 yrs.	BPH	immed. postop	1.04	0.735
			3 hrs. postop	0.34	0.55
			6 hrs. postop	0.26	0.00
2. B.M.	68 yrs.	BPH	immed. postop	2.43	3.76
			3 hrs. postop	2.28	1.91
			6 hrs. postop	1.21	1.07
			24 hrs. postop	1.42	1.00
			48 hrs. postop	0.26	0.00
			72 hrs. postop	0.00	0.00

[illegible] contracture.

TABLE 6. *Citrate and aconitate values in autopsy tissue*

	Diagnosis	CA	AA	PM
		mg./gm.	*mg./gm.*	
A. Prepubertal males				
1. Newborn male		0.00	0.00	7 hrs.
2. Newborn male		0.00	0.00	6 hrs.
3. G.R. 9 yrs.	Leukemia	0.00	0.00	4 hrs.
B. Adult males				
1. W.M. 26 hrs.	Nephritis	1.40	0.39	10 hrs.
2. R.R. 39 yrs.	Leukemia	0.58	0.15	4 hrs.
3. M.K. 40 yrs.	CA pancreas	0.52	0.28	10 hrs.
4. A.P. 45 yrs.	Coronary	1.21	0.16	7 hrs.
5. J. S. 51 yrs.	Cirrhosis	0.146	0.48	20 hrs.
6. H. M. 61 yrs.	CA lung	1.84	1.19	19 hrs.
7. N. S. 63 yrs.	Leukemia	2.31	1.28	6 hrs.
8. H. B. 64 yrs.	CA lung	0.53	0.44	6 hrs.
9. A. B. 67 yrs.	Coronary	2.21	0.85	10 hrs.
C. Averages:				
Men below 50 yrs.		0.925	0.215	
Men over 50 yrs.		1.410	0.759	

DISCUSSION

From the data accumulated it is apparent that citrate synthesis in the human prostate is a distinguishing biochemical feature of the organ. The exact function of citric acid in the physiology of the prostate is obscure. It is found in high concentration in benign prostatic tissue as noted in our series of 77 specimens secured at operation.

Evidence is available from the work of Kalnitsky (1949)[27] and Schneider, Striebich and Hogeboom (1956)[28] that the enzymes necessary for the synthesis of citrate by the cell are present in the mitochondrial apparatus. In addition the latter authors concluded that citrate is actually synthesized exclusively in the mitochondrial system of the secretory cell.

The chief biochemical sources of citrate synthesis within the prostate are dependent on the intracellular oxidation of metabolic fuels, such as carbohydrate, fatty acids, and amino acids. Such oxidative reactions are probably mediated through the configuration of the citric acid cycle as described by Krebs.[9] In addition transamination reactions involving glutamic acid, found in high concentration in prostatic tissue, contribute to the synthesis of citrate.[29-31] The increased concentration of citrate in prostatic adenoma may well result from active metabolic synthesis plus nonutilization of the substance as noted by Barron and Huggins[3] but the latter concept is not well supported from biochemical data available at this time.

In prostatic cancer our preliminary studies have shown that citrate concentration is markedly reduced to absent in simple prostatic tissue homogenates. The data suggest that there is a serious interference in citrate synthesis in prostatic malignancy. Whether this phenomenon represents a significant alteration in the operation of the citric acid cycle in this type of neoplasm is problematical and worthy of further experimental investigation. The suggestion is made that prostatic citrate determinations may be of value in the detection of latent, unsuspected prostatic carcinoma where routine histological sections have failed to demonstrate malignancy. It is probable that biochemical alteration of prostatic tissue may well occur long before malignant changes are histologically obvious to the pathologist.

SUMMARY

The physiological role of citric acid and aconitic acid in the mammalian prostate is poorly understood.

In a series of 77 benign obstructive prostates, homogenates of tissue taken from the operative field contained an average of 2.08 mg. citric acid and 1.14 mg. aconitic acid per gram of prostate.

In prostatic carcinoma the citrate and aconitate values were distinctly lower than in the benign group, ranging from 0.00–0.882 mg. citric acid and 0.00–0.418 mg. aconitic acid per gram of prostatic tissue.

Simultaneous determination of citrate and aconitate from various lobes of individual prostates gave a fair degree of correlation.

Preliminary studies suggest that there is significant alteration in citrate synthesis in prostatic carcinoma.

It is possible that prostatic citrate and aconitate assays may be of value in the detection of latent or incipient carcinoma of the prostate.

The suggestion is made that biochemical alteration of prostatic tissue may significantly antedate histological or clinical evidence of malignancy.

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Effect of Polycarboxylic Acids on Blood Clotting.* (24292)

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The anticoagulant action of citrate has been known for more than 60 years(1). The similarity in molecular structure of isocitrate and aconitate to citrate suggested that these tricarboxylic acids might also interfere with the clotting mechanism. To test this hypothesis, isocitrate, aconitate and the sodium salts of other polycarboxylic acids were incubated with human blood. Of the acids tested, those with 3 free carboxyl groups were effective in prolonging the clotting time.

Methods. Two ml of venous blood from normal volunteers or from patients without known clotting defects was discharged into tubes containing a solution of 0.5 ml of the appropriate test substance and into control tubes charged with 0.5 ml of 0.85% saline. The tubes were stoppered, inverted several times and placed upright in a rack at room temperature. Clotting time was defined as the interval between termination of mixing and formation of a firm clot which remained at the bottom when the tube was completely inverted. The polycarboxylic acids, obtained from commercial sources,† were put into solution, neutralized to pH 7 (pH Hydrion paper) and made up to volume with distilled water or saline just prior to use. The citric acid content of the commercial *cis*-aconitic acid, *trans*-aconitic acid, dl-Na_3 isocitrate, and tricarballylic acid was found to be less than 0.05%

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† Citric acid was purchased from Baker Chemical Co.; *cis*-aconitic acid, dl-malic acid, fumaric acid, tricarballylic acid, malonic acid, dl-isocitric acid lactone and Na_3 isocitrate were purchased from the California Foundation for Biochemical Research, Los Angeles. The source of the other compounds tested was the Nutritional Biochemical Corp., Cleveland, Ohio. *Trans*-aconitic acid, recrystallized from the commercial preparation, was found to be 86% pure by chromatography(2).

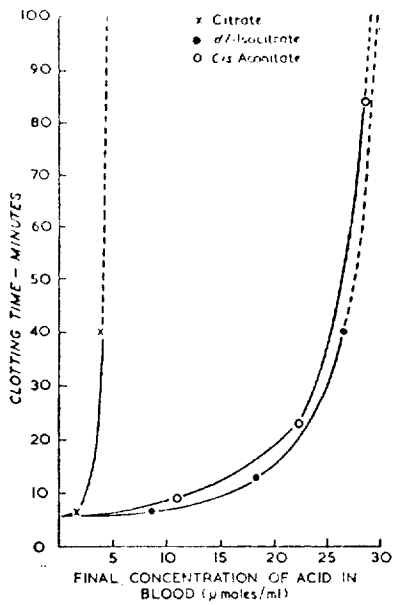

FIG. 1. Effect of varying concentrations of citrate, dl-isocitrate and *cis*-aconitate on clotting time of whole blood. Two ml of blood was added to 0.5 ml of a solution containing the test substance.

as determined by the method of Natelson, Pincus & Lugovoy(3). This amount of citric acid present as a contaminant would not appreciably influence blood clotting time.

Results. Both isocitrate and *cis*-aconitate were found to increase clotting time of whole blood (Fig. 1). The effect was dependent upon the concentration of polycarboxylic acid in the blood. Prolongation of clotting time was identical in the presence of equimolar concentrations of isocitrate and *cis*-aconitate. At a final concentration of 46 μmoles/ml both isocitrate and *cis*-aconitate increased clotting time to over 24 hours (Table I). The unnatural isomer, *trans*-aconitate, prolonged the clotting time, but was less effective than the *cis* isomer. Tricarballylate was also found to influence clot formation. The lactone of isocitric acid was relatively ineffective in increasing the clotting time. Only a slight prolongation of clotting time was noted when each of the dicarboxylic acids, α-ketoglutarate, succinate, fumarate, malate, glutarate, citraconate, glutamate and malonate was added to blood.

In other experiments, 2.0 ml of blood was mixed with 0.5 ml of 0.23 M *cis*-aconitate and permitted to stand for 4 hours. Control tubes contained 0.5 ml of saline and a drop of heparin (1000 U/ml) instead of *cis*-aconitate. At the end of the incubation period the cellular elements were removed by centrifugation, plasma proteins were precipitated with 3 volumes of 20% trichloracetic acid and the citric acid of the supernatant was determined. Plasma that had been incubated with *cis*-aconitate contained 5-7 μg citric acid/ml more than the control. This experiment indicated that a quantity of *cis*-aconitate, insufficient to affect clotting time, had been converted to citrate. Incubation of isocitrate in a similar manner did not reveal conversion of this acid to citrate.

Recalcification times were carried out on plasma from whole blood that had been incubated for 2 hours at room temperature with 0.046 M isocitrate and with *cis*-aconitate. Plasma clots formed between 5 and 10 min-

TABLE I. Effect of Polycarboxylic Acids on Clotting Time of Human Blood. 2 ml of blood mixed with 0.5 ml of 0.23 M acid (neutralized to pH 7). Control tubes contained 2 ml of blood plus 0.5 ml of 0.85% saline. Values are the means of a number (in parentheses) of experiments. Range of values is given beneath mean value in each case.

Acid	Clotting time	
	Control	Sample
Tricarboxylic	Min.	
Citric* (4)	5	>24 hr
Cis-aconitic (4)	5	>24 "
Trans-aconitic (4)	7	5–18 "
Isocitric (4)	6	>24 "
Isocitric lactone (4)	5	30 min.
		13–49 "
Tricarballylic (4)	5	>24 hr
Dicarboxylic	Min.	Min.
α-ketoglutaric (4)	5	10.5
		9–12
Succinic (4)	6	28
		18–41
Fumaric (4)	6	8
		7–9
Malic (7)	5	21
		17–42
Glutaric (4)	6	10.5
		4–15
Citraconic (3)	7	27
		26–29
Glutamic (2)	5.5	10
		8–12
Malonic (5)	5	12
		8–16

* Concentration .115 M.

utes after addition of 0.2 ml of 0.02 M $CaCl_2$ to 0.2 ml of plasma. Clot formation was not apparent 4 hours after $CaCl_2$ had been added to plasma from the tricarballylate incubated blood samples.

Discussion. These experiments indicate that blood clotting is inhibited *in vitro* in the presence of isocitrate and *cis*-aconitate as well as in the presence of citrate. None of the dicarboxylic acids tested was effective in prolonging clotting time; the lactone of isocitric acid was similarly ineffective. The mechanism of the inhibition has not been established. Although other interpretations are possible, it is likely that the tricarboxylic acids chelate with ionic calcium through the 2 terminal carboxyl groups as postulated by Heinz(4) to form relatively undissociable calcium complexes in the blood. Tricarballylate has been shown to complex with ionic calcium in pure solutions(5), but it appears from the recalcification experiments that its ability to prevent clotting stems from interference with the coagulation mechanism other than its Ca^{++} binding ability.

In the normal state it is unlikely that *cis*-aconitate or isocitrate play a significant role in prevention of blood clotting *in vivo* because of the small concentration of these substances in blood(6). It is possible, however, that these intermediates of the Krebs cycle might influence the intracellular concentration of ionized calcium. This concept would be especially important in those tissues, such as nerve and muscle, where small changes in concentration of ionized calcium are known to exert profound effects(7). Conversely, increased concentrations of ionized calcium in tissues might influence citric acid cycle metabolism by complexing with citrate, isocitrate or *cis*-aconitate thereby interfering with enzyme substrate formation.

Summary. Human blood was incubated *in vitro* with the sodium salts of a number of di- and tricarboxylic acids and their effect on clotting time was noted. *Cis*-aconitate, *trans*-aconitate, isocitrate and tricarballylate markedly increased clotting time of whole blood. Prolongation of clotting in the presence of isocitrate and *cis*-aconitate was dependent upon their concentration in blood. The possible mode of action of these metabolically active compounds and the implications of the findings were discussed.

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TRANS-ACONITATE UTILIZATION BY SHEEP

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Summary

Sheep fed diets containing 3·5 and 7·0% *trans*-aconitate on a dry weight basis for 5 days appeared normal and maintained normal levels of blood citrate, ketones, and aconitate, but showed large increases in urinary citrate. Calcium and magnesium levels in plasma and urine were not substantially modified. When *trans*-aconitate was placed in the rumen it disappeared rapidly but did not increase the concentration of rumen volatile fatty acids; blood and urinary aconitate values remained low. *trans*-Aconitate did not inhibit the fermentation of soluble substrates by rumen microorganisms *in vitro*. Both *cis*- and *trans*-aconitate were fermented slowly.

Intravenously injected sodium *trans*-aconitate at 1·0 m-mole/kg body weight produced no ill effects. The citrate which subsequently accumulated in blood and urine was not a radiometabolite of [1,5-^{14}C]*trans*-aconitate, suggesting that it was formed by aconitate hydratase inhibition. Plasma calcium and magnesium values were not depressed by intravenous *trans*-aconitate administration but urinary calcium excretion increased and urinary magnesium decreased. Under similar conditions of injection, sodium citrate was lethal.

These data are believed to exclude *trans*-aconitate as a sole cause of lethal aconitate hydratase inhibition or of hypomagnesaemia in sheep.

I. Introduction

Burau and Stout (1965) have suggested that *trans*-aconitate may poison cattle by forming magnesium ion complexes which induce hypomagnesaemia, or by competitively inhibiting aconitate hydratase, the enzyme catalysing interconversion of citrate, *cis*-aconitate, and isocitrate within the tricarboxylic acid cycle. These suggestions were prompted by the observation that high *trans*-aconitate concentrations occur in herbage from early spring pastures inducing hypomagnesaemia (Burau and Stout 1965). The highest *trans*-aconitate concentration (4·2% of dry weight) observed in a grass species was found in *Phalaris tuberosa* L. Symptoms resembling those of *Phalaris* poisoning in sheep have also been caused by dietary fluoroacetate, namely progressive citrate accumulation, loss of appetite, muscular incoordination, body tremors, tetanic convulsions, and death (Jarrett and Packham 1956). The biochemical basis for fluoroacetate poisoning appears to lie in its enzymic conversion *in vivo* to fluorocitrate (Peters 1957). Both fluorocitrate (Peters 1957)

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and *trans*-aconitate (Saffran and Prado 1949) act as competitive inhibitors of mammalian aconitate hydratase. *trans*-Aconitate has been previously shown to strongly inhibit the respiratory oxygen uptake by sheep rumen epithelium in tissue slices (Pennington and Sutherland 1956). It is therefore desirable to establish whether *trans*-aconitate can contribute to the *Phalaris* poisoning syndromes, currently ascribed mainly to the effects of tryptamine alkaloids (Gallagher, Koch, and Hoffman 1966).

Stout, Brownell, and Burau (1967) have found more than 1% *trans*-aconitate, on a dry weight basis, in 14 of the 30 grass species examined and in 8 of 54 dicotyledonous plants. Though there is clear need to study *trans*-aconitate metabolism by mammals, the action of *trans*-aconitate on rumen microorganisms is also obscure. Both inhibition (Pisano, Blahuta, and Mullen 1959) and utilization (Altekar and Rao 1963) have been demonstrated with bacteria. The present paper considers first the utilization of dietary *trans*-aconitate and then the effects of intravenously injected *trans*-aconitate on sheep.

II. Methods

(*a*) *Sheep and Materials*

The toxicity of *trans*-aconitate was tested with Merino or Merino × Border Leicester wethers weighing between 37 and 53 kg. Food was withheld from sheep during the 24 hr preceding injection or intraruminal administration experiments.

trans-Aconitic acid for feeding experiments was prepared from citric acid according to Bruce (1961) and recrystallized twice from acetic acid to achieve a melting point greater than 193°C (dec.). [1,5-^{14}C]*trans*-Aconitic acid was similarly prepared from [1,5-^{14}C]citric acid. *trans*-Aconitic acid, A grade, for injection experiments was obtained from California Corporation for Biochemical Research, Los Angeles. *cis*-Aconitic acid was synthesized from *trans*-aconitic acid according to the method of Deutsch and Phillips (1957). All acids were checked for purity by thin-layer chromatography as described later.

(*b*) *Analytical*

Unless otherwise stated, aconitate determinations were made according to Saffran and Denstedt (1948). Thin-layer chromatography on silica gel, using chloroform–methanol–formic acid (90 : 16 : 8 by volume) with *p*-dimethylaminobenzaldehyde as detecting agent (Smith 1960), was used for qualitative checks of *trans*-aconitate occurrence. Citrate was determined by a pentabromoacetone method (Stern 1957), modified in that the last traces of excess $KMnO_4$ were decolorized with 1·5% H_2O_2; interference by *trans*-aconitate was negligible. Blood ketones were measured as acetone according to Bakker and White (1957). Polyethylene glycol was determined by the turbidimetric method of Hyden (1956), taking the maximum reading after the addition of trichloroacetic acid reagent. Total volatile fatty acids were titrated after distillation in a Markham still (McClymont 1951).

Following intravenous injection with [1,5-^{14}C]*trans*-aconitate, blood and urinary organic acids were purified by partition on silica gel (Swim and Utter 1957), then applied in bands to 2-mm silica gel thin-layer chromatography plates and developed as described previously. After detection with a light spraying of *p*-dimethylaminobenzaldehyde reagent, the zones were scraped into scintillation fluid consisting of ethanol, dioxan, toluene, water (47 : 77 : 77 : 12 by volume) containing 80 g/l naphthalene and 5 g/l 2,5-diphenyloxazole, and counted in a scintillation spectrometer.

Calcium and magnesium were determined in urine collected into 1·0N HCl, and in blood plasma prepared and deproteinized as described by Wootton (1964). The determinations were carried out by atomic absorption spectroscopy methods (David 1960), modified in that ammonium

chloride was present in both sample and standard solutions. Strontium chloride (1500 p.p.m. Sr) in both sample and standard was used to suppress interferences in the determination of calcium.

(c) In vitro *Fermentation*

Samples of rumen fluid were obtained through rumen fistulae from three sheep, filtered through four layers of surgical gauze, and used either immediately or after periods of up to 2 hr at 38°C bubbled with carbon dioxide. Gas exchange accompanying organic acid fermentation was measured manometrically as described by McBee (1953) with 0·2 ml (20 μmoles) substrate and 0·8 ml rumen fluid. Flasks and manometers were flushed with carbon dioxide. The correction factor to overcome variation in flask volume was (gas volume of flask + manometer)/(gas volume of smallest flask + manometer). *In vitro* metabolism was further studied with rumen material from the same three rumen-fistulated sheep, which were fed 500 g lucerne pellets, pellets plus 0·2 mole citrate, or pellets plus 0·2 mole *trans*-aconitate respectively daily for 3 days. Incubations were made at 38–40°C in test tubes containing 0·5 ml (100 μmoles) substrate and 9·5 ml rumen fluid, bubbled with water-saturated carbon dioxide. Aconitate was determined spectrophotometrically, essentially as described by Racker (1950), on samples deproteinized with 0·1N sulphuric acid and centrifuged at 10,000 *g* for 30 min. Further measurements of aconitate disappearance during the course of *in vitro* fermentation were obtained, this time incubating the samples in McCartney bottles which were flushed with carbon dioxide, stoppered, and shaken at 38–40°C; aconitate was determined by the method of Saffran and Denstedt (1948).

(d) *Intra-ruminal Administration of* trans-*Aconitate and Citrate*

Approximately 500 ml rumen fluid was withdrawn, mixed with 2·5 g polyethylene glycol, 0·1 mole citrate or *trans*-aconitate (1·0M, one-third neutralized with NaOH), and returned to the sheep through a fistula. The experiment was done twice using only two sheep; thus each sheep received both *trans*-aconitate and citrate administration. Samples for volatile fatty acid, polyethylene glycol, and organic acid determinations were taken by withdrawing approximately 400 ml fluid by rumen pump, mixing, and sampling. Unused fluid was returned to the rumen. The sheep were fed lucerne pellets, both during the experiment and for several months beforehand.

(e) *Dietary Administration of* trans-*Aconitate and Citrate*

This experiment employed 10 sheep. Dietary additions were made by applying aqueous solutions of *trans*-aconitic acid or citric acid (one-third neutralized with NaOH) to lucerne pellets which were then dried at 70°C. The *trans*-aconitate additions, each administered for 5 days to two sheep, were 0·1 mole/day (approximately 3·5% *trans*-aconitate on a dry weight basis) and 0·2 mole/day (7·0% *trans*-aconitate). Another four sheep received similar treatments, except that citrate was substituted for *trans*-aconitate addition. Control analyses (Table 1) refer to analyses from the remaining two sheep which were each fed the same quantity of untreated pellets as the above sheep. Urine was collected with the aid of metabolism cages for one replicate of this experiment and then, to avoid food debris, into containers strapped to the sheep for the other replicate. Urinary aconitate values in Table 1 were obtained using the latter collection method. Urine was collected into 100 ml 1·0N HCl or into 20 ml 8% (w/v) thymol in isopropanol. Blood samples were taken by syringe from the external jugular vein and sodium heparin used throughout as an anticoagulant.

(*f*) *Intravenous Administration of Citrate and* cis- *and* trans-*Aconitate*

Intravenous injections were made with acids in 1·0M aqueous solution adjusted to pH 7·4 with NaOH. Injections were made over a 10-min period into an external jugular vein and post-injection blood samples withdrawn through a polyethylene catheter in the corresponding vein on the opposite side. *trans*-Aconitate at 1·0 m-mole/kg was administered to five sheep, *cis*-aconitate and citrate to one sheep each. A lower level of citrate, 0·3 m-mole/kg, was administered over 20-min periods to two sheep.

III. Results

(*a*) In vitro *Utilization*

The manometric readings in Figure 1(*a*) represent not only the carbon dioxide released from substrate and buffer, but also the production of other gases, chiefly methane. Glucose and citrate showed rapid rates of fermentation by rumen micro-organisms *in vitro*, which were not depressed by *trans*-aconitate [Fig. 1(*a*)]. Under these conditions *cis*- and *trans*-aconitate were fermented slowly. Citrate fermentation did not show a strongly developed initial lag phase, of the type observed by Clarke and Meadow (1959) to accompany citrate fermentation by *Pseudomonas aeruginosa* and attributed to adaptive permease formation.

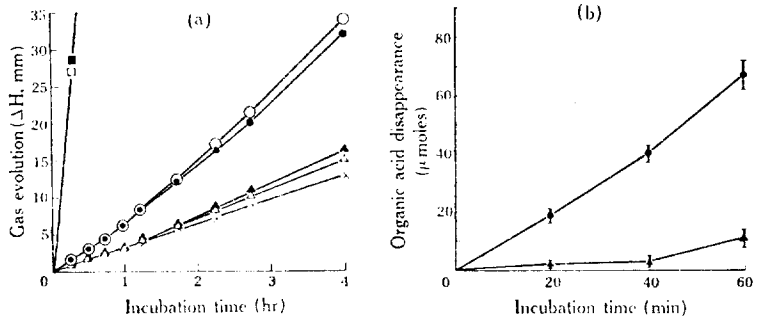

Fig. 1.—(*a*) Effect of *trans*-aconitate on the fermentation of soluble substrates. Gas volume expressed in terms of change in manometric height, ΔH. 0·8 ml rumen fluid incubated with 0·2 ml (20 μmoles) each of *cis*-aconitate (△), *trans*-aconitate (▲), citrate (●), and citrate+*trans*-aconitate (○). × No substrate. Results for similar experiments with glucose (□) and glucose+*trans*-aconitate (■) are also shown. (*b*) *In vitro* fermentation of citrate (●) and *trans*-aconitate (▲) by rumen fluid obtained from sheep fed diets supplemented with 3·5% citrate or 3·5% *trans*-aconitate for 3 days. 9·5 ml rumen fluid incubated with 0·5 ml (100 μmoles) substrate. Mean values plus standard error of means from six determinations are given.

Citrate, but not *cis*- or *trans*-aconitate, was substantially broken down during *in vitro* incubation with rumen fluid for 1 hr [Fig. 1(*b*)]. There appeared to be considerable variation in the rate of citrate breakdown by rumen fluid obtained from different sheep. In this experiment, disappearance rates were also measured at daily intervals over a 3-day period when sheep were fed organic acid supplements at 0·2 mole per day. The rate of *trans*-aconitate fermentation remained at the low level shown in Figure 1(*b*). However, the citrate breakdown on successive days, under similar conditions to those specified for Figure 1(*b*), was 44, 39, 56, and 66 μmoles per hour. A slow change of this type could be caused by altered microbial populations in the rumen, or adaption by existing types.

(*b*) *Intra-ruminal Administration of* trans-*Aconitate and Citrate*

Both citrate and *trans*-aconitate disappear rapidly from the rumen (Fig. 2). Citrate, as has been previously reported by Packett and Fordham (1965), increased the volatile fatty acid content of the rumen; *trans*-aconitate did not (Fig. 2). Follow-

ing administration of 0·1 mole of *trans*-aconitate into the rumen, the level of blood aconitate and citrate increased slightly from base values of 9 and 19 μg/ml respectively to achieve maximum values of 23 and 27 μg/ml 1 hr after administration.

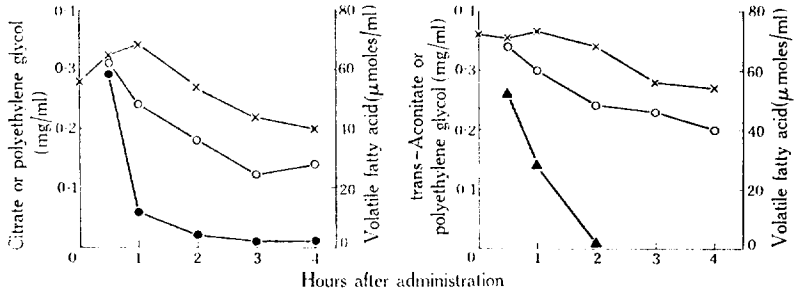

Fig. 2.—Comparison between curves for the disappearance from the rumen of added citrate (●) and *trans*-aconitate (▲) with that of polyethylene glycol (○) *in vivo* with rumen-fistulated sheep. Rumen volatile fatty acids (×) were also measured. Each point is the mean of values from two sheep.

(c) *Dietary Administration of* trans-*Aconitate and Citrate*

Sheep fed *trans*-aconitate appeared normal and had blood citrate and ketone values approximating those of control sheep (Table 1). However, urinary citrate

TABLE 1

EFFECT OF FEEDING CITRATE OR *trans*-ACONITATE SUPPLEMENTS TO SHEEP FOR 5 DAYS ON LEVELS OF BLOOD AND URINE COMPONENTS

Blood samples taken 1 hr after feeding. Aliquots of 24-hr sample of urine used for analysis. Urine collection commenced on the fifth day after feeding. Mean values are given for the two control sheep and for the four citrate-fed sheep. Separate values are given for each of the two *trans*-aconitate-fed sheep for blood samples and for the urine collected by the two different methods (see text). However, aconitate data for urine collected from metabolism cages are omitted

Daily Dietary Addition (mole)	Whole Blood			Plasma		Urine			
	Citrate (μg/ml)	Aconitate (μg/ml)	Ketone (μg/ml)	Mg (p.p.m.)	Ca (p.p.m.)	Citrate (μg/ml)	Aconitate (μg/ml)	Mg (p.p.m.)	Ca (p.p.m.)
None (control)	19	7	18	17	111	48	103	190	19
trans-aconitate									
0·1	22	12	9	16	118	520		260	9
0·1	24	15	12	17	110	420	180	254	13
0·2	27	17	21	17	113	660		310	15
0·2	24	7	22	18	105	805	240	172	13
Citrate									
0·1, 0·2	17	4	14	19	115	42	61	230	9

was greatly increased by the administration of *trans*-aconitate (Table 1). Subsequent trials with sheep fed 0·1 mole *trans*-aconitate per day have shown that within 24 hr the urinary citrate reached high values (approximated by those in Table 1) which

were maintained over the duration of the feeding trials. On the other hand, concentrations of aconitate in the urine were low (Table 1) and the urinary recovery was calculated as less than 1%. There was no marked influence on either plasma or urinary calcium and magnesium values (Table 1).

(*d*) *Intravenous Injection*

No abnormal behaviour was observed in the five sheep injected intravenously with *trans*-aconitate at 1·0 m-mole/kg body weight. These injections caused varying increases in the blood citrate level [Fig. 3(*a*)]. The blood volume was not measured

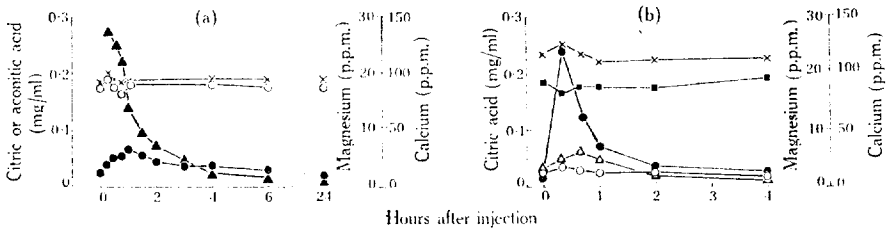

Fig. 3.—(*a*) Effect of intravenous *trans*-aconitate injection on blood citrate (●), *trans*-aconitate (▲), plasma magnesium (○), and plasma calcium (×) levels. Each point is the mean of values from three sheep. (*b*) Blood citrate levels following citrate (◕), *cis*-aconitate (△), and saline (●) injections. Plasma magnesium (■) and calcium (×) levels following citrate injection are also shown.

but, if it is assumed to be approximately 3 litres (from the data of Panaretto 1964), then only 9% of the injected *trans*-aconitate can be accounted for in the blood volume 20 min after the commencement of injection. The level of *trans*-aconitate was observed to fall rapidly [Fig. 3(*a*)] and there appeared to be rapid renal clearance into the urine (Fig. 4). The blood and urinary citrate was not a metabolite of

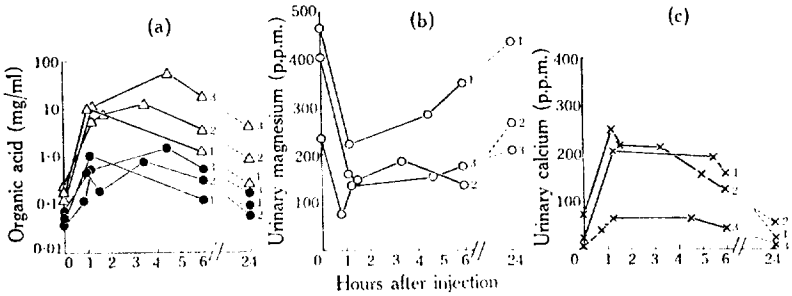

Fig. 4.—Effect of intravenous *trans*-aconitate injection on the urinary concentration of citrate (●), *trans*-aconitate (△), magnesium (○), and calcium (×) levels. Sheep 1 and 2 were fed lucerne pellets, sheep 3 poor quality *Phalaris* straw.

[1,5-^{14}C]*trans*-aconitate (Table 2). Approximately 40% of the injected *trans*-aconitate was recovered in the urine of the five sheep over a period of 24 hr.

Whereas the injection of 1·0 m-mole/kg of *trans*-aconitate did not result in any abnormal behaviour, injection of citrate at the same level killed one animal within 7 min. Injections of citrate at 0·3 m-mole/kg over a 20-min period appeared to be close to the toxic dose and caused high urinary citrate and calcium excretion without marked change in urinary magnesium excretion (Table 3). A similar but less pronounced rise in calcium content was apparent in the urine of sheep injected with *trans*-aconitate (Fig. 4); plasma magnesium and calcium levels remained approximately constant following injection [Fig. 3(*a*)]. From stability constants (Sillen and Martell 1964) it can be calculated that at pH 7·4 citrate has a much higher binding power for Ca^{2+} than for Mg^{2+} and Mn^{2+} ions. Following citrate injection, plasma calcium levels were not greatly modified [Fig. 3(*b*)].

TABLE 2

RADIOACTIVITY OF *trans*-ACONITATE AND CITRATE IN BLOOD AND URINE FOLLOWING INTRAVENOUS INJECTION OF [1,5-^{14}C]*trans*-ACONITATE TO SHEEP 2

Specific activities, expressed as disintegrations per minute per 100 μg, given in parenthesis

Time after Injection (hr)	*trans*-Aconitate in Blood (μg/ml)	*trans*-Aconitate in Urine (μg/ml)	Citrate in Blood (μg/ml)	Citrate in Urine (μg/ml)
0·7	206(4,031)		85(0)	
1·0	95(3,537)		56(31)	
3·5		12,800(3,621)		794(28)
6·0		3,400(3,745)		305(41)

TABLE 3

URINE COMPOSITION FOLLOWING SODIUM CITRATE INJECTION

1·0M sodium citrate, pH 7·4, was injected into two sheep at the rate 0·3 m-mole/kg body weight

Time from Injection to Sampling	Volume (ml)	Citrate (μg/ml)	Ca (p.p.m.)	Mg (p.p.m.)
Sheep 1				
Preceding 24 hr	1016	38	19	235
0·25–0·5 hr	180	2800	216	128
0·5–3·0 hr	50	2400	70	105
Sheep 2				
Preceding 24 hr	1160	43	32	235
0·3–1·2 hr	60	1500	80	121
1·2–2·2 hr	100	814	67	108
23–25 hr	90	30	30	95

IV. Discussion

Sheep fed *trans*-aconitate supplements appeared normal and had blood citrate values within the normal range. The slow rates of *trans*-aconitate metabolism observed *in vitro*, coupled with rapid *trans*-aconitate disappearance from the rumen *in vivo*, suggest rapid *trans*-aconitate absorption through the rumen epithelium. Subsequently, blood and urinary aconitate values remained low, further indicating that *trans*-aconitate was utilized mainly within sheep tissues. The unexpectedly low urine recoveries of *trans*-aconitate observed in these experiments suggest a need to study the probable metabolism of *trans*-aconitate by animal tissues, where appropriate enzymes attacking this compound have not so far been found.

The sheep responded to dietary *trans*-aconitate by increased urinary citrate excretion. The citrate excreted following injection of [1,5-^{14}C]*trans*-aconitate was not a radiometabolite of the latter (Table 2) and is therefore suggested to have been formed by aconitate hydratase inhibition, probably in tissue not metabolizing *trans*-aconitate. It should be noted, however, that Pennington and Sutherland (1955) have shown that the respiratory oxygen uptake by rumen epithelium was stimulated by *cis*-aconitate and the other Krebs cycle acids at a concentration (10 μg/ml) which permitted *trans*-aconitate to exert powerful inhibition. Failure of *trans*-aconitate, in present circumstances, to cause a lethal metabolic block can be partly attributed to weak aconitase inhibition (Thomson *et al.* 1966), rapid urinary clearance (Fig. 4), or perhaps failure to reach mitochondrial aconitate hydratase.

It is difficult to account for the way dietary *trans*-aconitate increased urinary citrate excretion (Table 1). The low citrate levels in the blood (Table 1) contrast with the high levels observed in sheep poisoned with fluoroacetate (Jarrett and Packham 1956). Data are not available concerning the urinary citrate excretion of fluoroacetate-poisoned sheep. It is known, however, that urinary citrate remains unchanged despite greatly increased tissue and mitochondrial citrate in fluoracetate-poisoned rats (Crawford 1963). Conversely, intravenously injected analogues of Krebs cycle acids have caused approximately 25-fold increases in the urinary citrate of the dog (Orten and Smith 1937), without increasing blood, liver, or muscle citrate (Orten and Smith 1939). Thus, in the present instance, *trans*-aconitate may have induced a renal, rather than a whole body response, resulting in rapid citrate elimination in the urine. The mechanisms controlling renal citrate disposal are ill defined but acid-base balance, active reabsorption, or other causes could be invoked. The kidney is also particularly active in citrate synthesis (Orten and Smith 1939) and utilization (Herndon and Freeman 1958). Possibly *trans*-aconitate may preferentially accumulate in renal tubular cells and cause enhanced aconitase inhibition there. Citrate levels are normally extremely high in kidney tissue following fluoroacetate poisoning (Peters 1957).

In the present experiments added citrate disappeared from the rumen fluid *in vitro* (Figs. 1 and 2). Citrate may also have passed through the rumen epithelium but, if this were so, the low values obtained for blood and urinary citrate indicate rapid citrate utilization in the body, perhaps via the citrate cleavage enzyme recently thought to be associated with lipogenesis (Ballard and Hanson 1967). The toxicity of citrate injected intravenously has been long known (Salan and Wise 1916) and

can be abolished by calcium salt injections (Krebs, Savlin, and Johnson 1937), suggesting that citrate binds calcium ions, causing hyperirritability and, ultimately, heart block. The present data indicate that citrate injection causes rapid calcium excretion without raising urinary magnesium levels. On the other hand, comparatively high doses of intravenously injected *trans*-aconitate modify plasma calcium and magnesium values only slightly [Fig. 3(*a*)]; nor do these high doses induce hypomagnesaemia but, like citrate injections, increase the urinary calcium level without increasing that of magnesium (Fig. 4; Table 3).

Tolerance of the sheep towards *trans*-aconitate was so great that, despite the short duration of these experiments, it is highly unlikely that *trans*-aconitate, by itself, could be a major factor inducing hypomagnesaemia or lethal metabolic inhibition in sheep.

V. Acknowledgments

Atomic absorption determinations of calcium and magnesium were very kindly performed by Dr. D. J. David. Acknowledgment is made of the advice of Dr. M. Freer, Mr. K. R. Christian, and Professor C. H. Gallagher. Messrs. M. D. Holgate and T. Shepherd gave skilled technical assistance.

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ORGANIC ACIDS AND GRASS TETANY (1)

Part One

by

F. Lomba, G. Chauvaux and V. Bienfet (2)

General Introduction

A drop in blood magnesium is generally produced during the passage of cows from their winter diet to the rapidly growing grass of spring, especially if there has been intensive use of nitrogen (Bartlett et al. 1954 - Ender, Disnington and Helgebostad 1957), and more especially of nitrogen and potassium (Kemp 1962 - Smyth, Conway and Walsh 1958 - Hvidsten et al. 1959). In order to explain this hypomagnesemia, the blame has been placed, among other things, on the botanic composition of the prairie ('t Hart 1960), the mineral composition of the spring grass (Kemp and 't Hart 1957), or even on the low digestibility of the magnesium which it contains (Kemp 1962 - Rook and Storry 1962 - Dishington 1964 - Care et al. 1967). If the study of all these factors has been able to lead to the establishment of excellent preventive measures, recently well defined by Burns and Allcroft (1967), it still remains that, despite the works of numerous researchers, cited by Liegeois and Derivaux (1962), the alterations of the magnesium of the animals are insufficiently understood. However, we think that they must be important, whether it is created in the rumen of the conditions (pH, complexive substances...) which the blood magnesium flees from to the content of the rumen or where it is retained, whether there is, under the effect of spring grass, significant digestive elimination of the endogenic magnesium, or whether there is chelation of the blood magnesium by substances capable of passing from the rumen into the blood.

The research which we have been conducting has always aimed at realizing conditions in agreement with these conceptions. Thus we have administered an excess of proteins or even amino acids, of different salts, or pomes... In certain cases we have arrived at determining sudden hypomagnesemias, but the results were too inconstant or of too short duration to be of any significance.

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It is at this stage of our investigations that we have received word of observations made by the American researchers Stout and Burau (1965 and 1967) and Bohman (personal communication). They immediately seemed to us to be worthy of interest. In effect, Burau and Stout (1965), studying the composition of tetany prairie grasses of California, discovered that organic acids could attain very elevated volume in the plants of these prairies. In certain cases they found up to 3% trans-aconitic acid in the dry matter. The presence of this acid itself is surprising, for it is its isomer, cis-aconitic acid, which one expects to find in these plants: it is the latter which is associated with aconitase in the citrate -- isocitrate transformation of tricarboxylic acid (Conn and Stumpf 1964). Burau and Stout, pursuing their investigations, soon formulated the hypothesis (Burau and Stout 1965 - Stout, Brcwnell and Burau 1967) that these organic acids could contribute to the development of grass tetany. Starting from this hypothesis, Bohman (personal communication), of the University of Nevada, provoked the appearance of disturbances or deaths which he compared to those of a case of tetany. In order to do this, he administered to cows, in a single oral dose, 500 g of an organic acid -- citric or trans-aconitic -- and 500 g of potassium chloride. The absence of data on the evolution of calcemias and magnesemias of cows thus treated is to be regretted in these experiments. Nonetheless, these works were of very high interest and it seemed important to us to confirm them, especially as other researchers, in England in the United States, have already found that organic acids can influence magnesemia. This is the case with Burt and Thomas (1961), who obtained a significant decrease in the blood magnesium and phosphorus by feeding heifers on diets containing 1% citric acid. It is too bad, however, that in a too brief description of the ration, they do not give its mineral composition, and neglect certain important elements, such as the level of proteins. For their part, Camp and Dollahite (1967) attribute to the introduction of trans-aconitic acid into the rations the power of lowering magnesemia in the sheep.

The investigations which we have since undertaken had as a goal the specification of the possible manner of action of the organic acids: their persistance in the rumen, their passage from the rumen to the blood, at what rate and under what form. Were they revealed to be capable or not of inducing hypomagnesemia and possibly hypocalcemia, and could they thus intervene in the determination of grass tetany? In order to attempt to specify the disturbances described by Bohman, we attempted to reproduce them with the help of mixtures of organic acids and potassium chloride. Finally it was necessary to see whether our spring grasses contain quantities of these organic acids as high as those of the californian grasses.

Our activity was thus divided as follows:

1. Using rabbits, we tested whether and in what way the organic acids influenced plasmatic calcium and magnesium. This is the subject of this first part.
2. From 4/14/1967 to 5/19/67, we studied the acid content of our spring grasses on 8 farms where numerous cases of tetany had appeared in preceding years. The results of this inquest will be published in a second part.

3. We studied that which occurs in cows receiving significant amounts of citric acid or trans-aconitic acid and potassium chloride in a single dose directly in the rumen. The third part will be devoted to the results thus obtained.
4. Finally, we administered these acids several times during prolonged periods to cows, in order to see whether or not they induced hypomagnesemia or hypocalcemia. In the fourth part, we study the effects of such administration.

Part One

EFFECTS IN THE RABBIT

The present work gives the essential observations which we were able to make upon continually perfusing trans-aconitic acid intravenously to rabbits. We compared this action to that of oxalic and citric acid, which are better known in biology (Shelling and Maslow, 1928. Watts 1956 and 1959. Safran and Denstedt 1951. Kwatra and Kera 1965) and to that of pyruvic acid, the complexive power of which is better known (Neuman and Neuman 1958), as well as its production and arrival in the rumen (Van Der Horst 1965).

Materials and Methods

Perfusion:

Realized with the Brown perfusor, the flow of which is regulated by injection 0.5 ml of solution per minute.

Products injected:

One single attempt was made with a simple solution of acid in water.

All the other solutions were prepared with a pH of 7.3 with sodium before dilution and utilization.

Thus we studied:

pyruvic acid at 2.5 and 10%
trans-aconitic acid at 1.5 and 10%
citric acid at 2 and 5%
oxalic acid at 0.5 and 2%
2 mixtures of acids, A and B

Mixtures A and B reproduce the proportions of organic acids in our grasses, which contain at most:

	in mval		in mval
fumaric acid	9.09	trans-aconitic acid	21.95
succinic acid	9.07	malic acid	59.54
malonic acid	10.91	citric acid	47.59
oxalic acid	2.60		

Since we did not have enough malonic acid, we replaced it in mixture A with 9.01 mval of oxalid acid, and in mixture B with 9.79 mval of trans-aconitic acid.

Manner and time of withdrawal of blood samples

The blood was withdrawn:
-- partly from the vein of the ear, at the beginning of each experiment before commencing the perfusion on the already attached rabbit.

-- partly at the end of each experiment, this end being either the death of the animal -- and in this case the blood was pumped directly from the heart -- or decided because the volume injected (see table I) became too great and produced not the least disturbance.

The withdrawal was made with a heparinated syringe. The blood was immediately tranferred without contact with the air, under liquid paraffin, into centrifuging tubes, so that all gaseous exchange with the air was avoided, an exchange which would cause modifications of the pH and thus of the equilibrium among the various forms of calcium and magnesium (Laviette 1937 - Ludwig et al. 1942). After centrifuging at atmospheric temperature, the plasma was aspirated and conserved under paraffin (Breen and Freeman 1961 - Carlstrom 1955 - Munday and Mahy 1964).

Measurement of the ultracentrifugable fraction of calcium and magnesium

The works of Van 't Klooster (1967) and of Ludwig et al (1942), Canutin et al (1942), allow for the confusing of the ultracentrifugable fraction with the ultrafiltrable fraction. The dosage of proteins is necessary in order to establish the exact percentage of the ultrafiltrable fraction, since the calcium and magnesium are given in concentration in the plasmatic water. It is done according to the method of Munday and Mahy (1964). The quantity of plasmatic water is given by the formula of MacLean and Hastings (1935)

$$A = \frac{99-0.75.P}{100}$$

in which A is the percentage of plasmatic water and P the concentration in g/100 of proteins.

Thus

$$C\ plasma = A.C\ ultrafiltrable$$

where C plasma is the concentration in the plasma and C ultrafiltrable the concentration in the ultrafiltrate.

Ultracentrifugation

We centrifuge at 60,000 turns per minute (25,000 g) for 6 hours under paraffin in an Internation ultracentrifuge, model B 60, with angular cap at 10°C, the temperature not having influence between 5.50°C and 30°C on the ultrafiltrable fraction (Breen and Freeman 1961).

Dosage of calcium and magnesium

By means of spectrophotometry of atomic absorption Perkin-Elmer, model 303, according to the methods described by Willis

Table I

	A. Acide injecté	B. Quantité injectée en mval	B. Quantité injectée mval/Kg	C. mval/L de plasma a Ca avant	C. mval/L de plasma b Ca après	C. mval/L de plasma a Mg avant	C. mval/L de plasma b Mg après	D. pourcentage ultrafiltrable a Ca avant	D. pourcentage ultrafiltrable b Ca après	D. pourcentage ultrafiltrable a Mg avant	D. pourcentage ultrafiltrable b Mg après	E. Mort	F. Convulsions
1.	Pyruvique												
	5,0%	60,34	24,09	3,19	3,89	3,15	2,13	-	-	-	-	-	-
	2,5%	13,99	10,20	7,03	6,40	2,37	2,23	64,7	78,1	73,9	80,1	-	-
	2,5%	29,90	14,30	5,29	6,48	1,01	1,31	-	71,6	-	96,8	-	-
	10,0%	33,24	23,08	5,74	6,41	1,76	1,98	-	77,6	-	87,7	-	-
	10,0%	44,03	24,32	5,99	8,78	1,48	1,69	55,5	91,2	44,7	90,0	+	+
2.	trans aconitique												
	1,0%	64,45	17,40	6,04	9,08	5,36	3,85	-	-	-	-	-	-
	5,0%	78,06	22,75	6,19	5,79	4,37	3,55	-	-	-	-	-	-
	10,0%	139,24	60,83	4,40	5,49	3,68	6,31	-	-	-	-	+	+
	5,0%		18,61	6,29	5,89	2,70	2,40	67,9	75,2	68,3	80,1	-	-
	5,0%	24,99	24,99	5,19	6,09	1,77	1,12	-	72,0	-	89,7	-	-
	5,0%	36,0	36,36	3,52	7,31	2,19	4,52	-	69,0	-	42,7	-	-
	10,0%	159,40	88,07	6,49	7,58	2,05	2,14	76,1	88,8	78,4	46,7	+	+
3.	Citrique												
	5,0%	32,3	18,7	6,98	12,02	3,55	5,66	-	-	-	-	+	+
	5,0%	47,9	15,8	8,08	10,96	5,51	6,04	-	-	-	-	+	+
	2,0%	9,29	6,15	5,79	7,01	1,43	2,23	72,7	86,4	51,7	68,9	+	+
	2,0%	10,07	9,15	6,11	7,41	2,31	3,31	68,3	86,2	58,0	73,4	+	+
	5,0%	5,73	4,24	5,46	5,76	2,14	3,37	62,5	80,4	49,2	68,8	+	+
	5,0%	11,12	6,70	6,14	7,01	1,31	1,96	59,4	89,3	65,6	75,7	+	+
4.	Oxalique												
	0,5%	2,58	1,45	6,19	0,80	1,45	1,74	69,7	71,9	78,9	79,2	+	+
	2,0%	3,20	1,55	5,61	1,35	1,96	2,69	75,6	30,0	72,8	80,4	+	+
	2,0%	2,73	2,31	6,79	0,50	2,58	7,28	68,5	91,0	63,4	23,6	+	+
5.	Mélange A												
	10,0%	57,94	47,88	6,69	5,64	4,49	5,36	71,0	93,7	57,5	82,0	+	+
	10,0%	55,39	37,17	4,19	4,39	3,45	5,52	86,1	100	52,8	68,9	+	+
6.	Mélange B												
	10,0%		60,62	6,34	9,28	2,53	3,90	69,6	87,2	71,7	83,7	-	-
	10,0%	91,0	56,17	-	8,75	-	2,55	-	84,4	-	76,1	+	+

Key:
A.= acid injected B.= amount injected C.= mval/L of plasma
D.= ultrafiltrable percentage E.= death F.= convulsions
a.= before b.= after
1.= pyruvic
2.= trans-aconitic
3.= citric
4.= oxalic
5.= mixture A
6.= mixture B

(1960a, 1961) for the calcium, and by Willis (1960b), Dawson and Heaton (1961) and Stewart et al. (1963) for the magnesium.

Results and discussion

The intital calcemias, which range from 3.19 to 8.08 mval/L with an average of 5.82 mval/L, are comparable to those measured by Sheahan and Maslow (1928), Cole et al (1944), Safran and Denstedt (1951): from 5.19 to 9.8 mval/L, with an average of 7.54 mval/L.

The magnesemias, which vary between 1.01 and 5.51 mval/L with an average of 2.58 mval/L are in general more elevated than those which Hunkel et al (1947) give; from 1.66 to 2.40 mval/L with an average of 1.98 mval/l, or than those which we ourselves obtained in a large number of rabbits: from 1.22 to 3.35 mval/L, with an average of 2.14 mval/L. However, there is no significative difference (t = 0.546) between these two groups of results.

Toxicity of injected acid

From this point of view there exist great differences between the acids used. These differences are due at once to the quantity, expressed in mval/kg of live weight, necessary to kill, and the rapidity with which death occurs. On the other hand, each time that it was produced, death was preceded by the same manifestations: after a very short or longer delay, according to the acid, its concentration and even the speed of the injection, we noted the appearance of convulsions, death following after an equally shorter or longer interval.

In table II, we reproduce the lethal quantities, the interval between the beginning of the perfusion and the first appearance of the convulsions and finally the time which has passed between this moment and death.

Table II

	A. Acide	B. mval/Kg de poids vif nécessaire pour tuer	C. Apparition de convulsions après (en min)	D. Durée de la période des convulsions (en min)
1.	Oxalique			
	0,5%	1,45	8	44
	2%	2,31	2	6
2.	Citrique			
	2%	6,15 - 9,15	60	30
	5%	4,24	15	10
	5% (injection lente)	6,70	60	60
3.	Trans aconitique			
	10%	60,80	160	3
	10%	88,70	35	54
4.	Pyruvique			
	10%	24,32	60	30

Key:
A.= acid B.= mval/kg of live weight necessary to kill
C.= appearance of the convulsions after (in min)
D.= duration of the period of convulsions (in min)
1.= oxalic 2.= citric 3.= trans-aconitic 4.= pyruvic

Mixture A, which is the one which contains the most oxalic acid, kills at 27 and 47 mval/kg, while mixture B, where the oxalic acid is replaced by trans-aconitic acid, only kills with about 60 mval/kg.

A classification of these acids, in decreasing order of toxicity, puts oxalic acid in first place. In order to obtain the same result, one needs about three times as much citric acid, eleven times pyruvic acid, and thirty times as much trans-aconitic acid.

Here we observe a first and important difference between trans-aconitic acid on the one hand and citric and oxalic acid on the other: in comparison with these latter, trans-aconitic acid is only very slightly toxic. Table II also shows that the concentration and speed of the injection of the acid play an important role:

-- 2% oxalic acid kills in 8 minutes, while it took 52 minutes for 0.5% oxalic acid. It is true that in this case, the amount perfused is less.

-- 5% citric acid in a slow injection, at a dose of 6.7 mval/kg kills in 120 minutes, while in a 5% solution, in a more rapid injection, it kills at a dose of 4.24 mval/kg and in 25 minutes.

-- In a 2% solution, the rabbit tolerates as much as 9.15 mval/kg and takes 90 minutes to die.

Action on the ions

a. On the total calcium

Oxalic acid causes a nearly complete disappearance of the calcium.

Citric and trans-aconitic acid, on the contrary, increase calcemias, citric acid significantly ($p < 0.05$) and trans-aconitic acid less so ($p < 0.1$).

Pyruvic acid does not modify the calcium significantly

b. On the total magnesium

The only significant modification obtained is with citric acid, which has the effect of increasing the total magnesium ($p < 0.01$).

Oxalic acid also increases magnesemias, but insignificantly so, while trans-aconitic acid and pyruvic acid do not modify the total magnesium.

c. On the ultracentrifugable fraction of these ions

On this level also we observe important divergences in the action of the acids. If we first study the percentage of the ultrafiltrable in relation to the total, we observe that oxalic acid does not significantly modify the percentage of diffusible calcium; trans-aconitic acid increases it, but not significantly ($p < 0.2$), while pyruvic acid and citric acid especially increase it significantly: $p < 0.05$ and $p < 0.02$.

As concerns the percentage of diffusible magnesium in relation to the total magnesium, only citric acid increases it in a significant fashion ($p < 0.01$), while it is not significantly modified by the other acids.

However, the diffusible calcium and magnesium are more modified than appears from the study of the percentages, as is shown in table III.

Table III

	A. Acide injecté	B. Concentration en ultracentrifugable en mval / L			
		a. Calcium	b.	a. Magnesium	b.
		Avant	Après	Avant	Après
1.	Pyruvique 2,5%	4,79	5,04	1,84	1,88
	10%	3,34	6,11	0,64	1,86
2.	Trans aconitique 5%	4,49	4,64	1,94	2,01
	5%	-	4,59	-	1,05
	5%	-	4,29	-	2,02
	10%	5,19	6,99	1,69	1,03
3.	Citrique 2%	4,42	6,36	0,78	1,60
	2%	4,39	6,71	1,41	2,56
	5%	3,59	5,39	1,11	2,42
	5%	3,83	6,54	0,90	1,55
4.	Oxalique 0,5%	4,59	0,60	1,22	1,45
	2%	4,47	0,42	1,50	2,27
	2%	4,89	0,47	1,72	1,80

Key:
A.= acid injected B.= concentration in ultracentrifugable in mval/L
a.= before b.= after
1.= pyruvic
2.= trans-aconitic
3.= citric
4.= oxalic

With the exception of oxalic acid, which significantly decreases it, the acids increase the concentration of ultracentrifugable calcium in a significant fashion. In the same way they significantly increase the diffusible magnesium. We were unable to appraise the action of trans-aconitic acid in this respect.

d. On the fraction bound to proteins

The results are collected in table IV.

Table IV

	A. Acide	B. Quantité liée aux protéines en mval / L de plasma			
		a. Calcium	b.	a. Magnesium	b.
		Avant	Après	Avant	Après
1.	Pyruvique 2,5%	2,48	1,41	0,62	0,45
	10%	2,02	1,49	0,82	0,17
2.	Trans aconitique 5%	1,56	0,85	0,86	0,48
	10%	1,50	1,07	0,45	1,15
3.	Citrique 2%	2,01	1,30	0,71	0,70
	2%	2,05	0,60	0,98	0,89
	5%	2,50	0,70	1,09	1,06
	5%	2,50	0,70	0,46	0,48
4.	Oxalique 0,5%	1,80	0,30	0,31	0,37
	2%	1,37	0,90	0,54	0,53
	2%	2,14	0,05	0,95	5,57

Key to table IV:
A.= acid B.= amount bound to proteins in mval/L of plasma
a.= before b.= after
1.= pyruvic
2.= trans-aconitic
3.= citric
4.= oxalic

In the case of calcium, the fraction bound to proteins is reduced with all the acids, but especially with citric and oxalic. This once again distinguishes trans-aconitic acid from citric and oxalic acid.

For the magnesium, we obtained no significant modification of the fraction bound to proteins. On the other hand, we do not sufficiently understand the 5.57 obtained with 2% oxalic acid.

****

The modifications caused by the acids studied of blood calcium and magnesium, as well as the fractions bound to proteins and the ultracentrifugable fractions, are shown in table V.

Table V

Calcium			Acide	Magnésium	
Total				Total	
Protein bound		Diffusible		Protein bound	Diffusible
+*	+***	+*	Oxalique	-	+
+*	+**	+*	Citrique	-	+**
+	+*	+*	Tr. Aconitique	-	+
+	-	+*	Pyruvique	-	+
*** très hautement significatif				+ augmentation	
** hautement significatif				- diminution	
* significatif					

Key:
*** very highly significant
** highly significant
* significant

This table shows that:

1. The calcium is more rapidly liberated by the proteins than the magnesium.
2. The action on the calcemia, whether it is a question of increase or decrease, is more marked than that exercised on magnesemia, citric acid being the only one to increase significantly the toal plasmatic magnesium.
3. From the point of view of intensity of action, trans-aconitic action, like pyruvic acid, is clearly distinguished from oxalic

acid and citric acid.

Conclusions

In the precise objective which is ours, that of studying the determination of hypomagnesemia, none of these acids seems to be able to be retained: except for the case of oxalic acid, there is in the rabbit no relation between the total blood levels or ultrafiltrable levels of calcium and magnesium, and the determination of death or the convulsive phenomena which precede it. Oxalic acid, very toxic, certainly causes the nearly complete disappearance of calcium, but it increases the magnesium on the other hand.

-- Citric acid influences neither the calcium nor the magnesium in the manner hoped.

-- Trans-aconitic acid, less toxic to be sure, is also less active on the alkaline-earth of the blood, which it elsewhere increases.

-- The same is true of pyruvic acid.

These preliminary experiments do however erase much of the possible interest in organic acids in the study of the determination of hypomagnesemial tetany.

Summary

The authors studied the action of several organic acids in the rabbit, paying particular attention to the modifications of calcium and magnesium. Trans-aconitic acid was shown to be very different from citric acid, and especially from oxalic acid.

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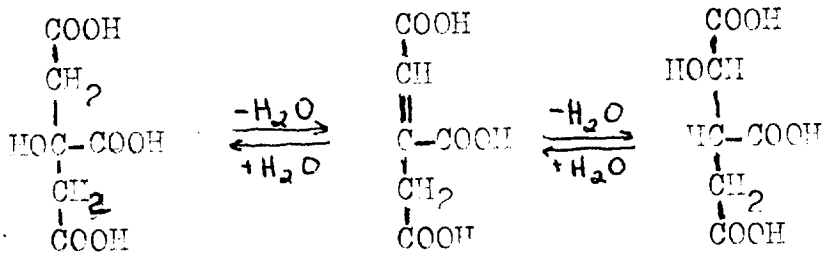
Kaiser Wilhelm Institute for Medical Research

Heidelberg, February 6, 1939

Chairman: Ch. Crundreen

C. Martius, Chairman: "Synthesis and Catabolism of citric acid in its importance to the problems of intermediary metabolism."

About three years ago, the question of whether the citric acid which occurs in a small measure in the animal body is of purely exogenous or endogenous origin was finally definitely answered by means of some balance experiments: It can be viewed as a normal metabolic product. As is know today, only such hydrogen atoms can be detached in enzymatic dehydration, as are separated from one another by two atoms (C -C C-O C-H). Since such hydrogen atom pairs are not present in the molecule of citric acid, citric acid must be surrounded by its dehydration. This would lead one to think of the appearance of an isocitric acid, which could formally be formed through aconit acid out of citric acid.



Citric acid Aconit acid Isocitric acid

Hitherto, isocitric acid was found in nature only in blackberry juice. It is easily acted on by citricodehydrase; the first catabolite is α-ketoglutaric acid. This acid was also isolated in the catabolism of citric acid. Synthetic isocitric acid is surrounded by suitable enzyme preparations in citric acid; after the expiration of the reaction upon the addition of molybdate, the preparation show intense turning, a sign that only an optically active form is reacting. The equilibrium is of 10% isocitric acid and 90% citric acid. Aconit acid is present in the equilibrium only in unmeasurable quantities. The common trans-aconit acid is not acted upon by the enzyme preparations; the labile cis-form, on the other hand, is hydrated. Thereupon, initially, equal amounts of citric and isocitric acid form, as is revealed from measurements of the turning, which reaches a maximum. In this way, the natural optically active citric acid can be easily obtained in larger quantities. The surrounding enzyme, aconitase, is widespread in nature and not identical with fumarase.

Citric acid can be obtained from a condensation product of oxaloacetic acid with pyruvic acid. It is formed in a weak alkali solution at room temperature. The increased citric acid excretion after injections of malic acid, fumaric acid and succinic acid suggests a similar course of the synthesis. The Krebs pattern, according to which the total carbohydrate catabolism passes the level of citric acid, is dismissed by the lecturer, especially because according to <u>Orten and Smith</u> citric acid originates only in the kidneys . -- The physiological significance of citric acid is in an entirely different area, the synthesis of the amino acids. Their catabolic product, ketoglutaric acid, can be easily -- now also enzymatically -- be converted to glutamic acid by the same enzyme that causes the dehydration of glutamic acid. This relatively most important amino acid serves further, in <u>Braunstein-Kritzmann</u> transamination , as the donator of the amino group; it is therefore also involved in the synthesis of other amino acids. -- The narrow relationships between citric acid and glutamic acid can be seen, for example, in the fact that glutamine-rich plant embryos also contain an abundancy of citric acid. A further confirmation of the proposed theory is offered by the observation, that the known citric acid formator Aspergillus niger requires quite a bit of oxygen and must grow in complete health, if it is to form citric acid. Thus here too we see the close relation between citric acid and amino acid synthesis. -- Since citric acid arises in carbohydrate catabolism, it forms a bond between this class and the proteins.

Colorimetric Determination of Aconitic Acid in Sorgo

William E. Poe and Ben F. Barrentine

In a rapid colorimetric method for the determination of aconitic acid in sorgo juice, the acid is extracted from clarified starch-free juice with 2-butanone after lowering the juice pH to 1.3. One extraction removes 99% of the aconitic acid. Other organic acids, at the level found in sorgo juice, do not interfere with color development. Acetic anhydride and pyridine, when added to an aliquot of the 2-butanone layer, produce a purple color which is read on a spectrophotometer at 550 mμ.

Aconitic acid (1,2,3-propenetricarboxylic acid) is found in sorgo juice in amounts ranging from 0.2 to 1.0%. Its presence is reported to give the characteristic bitter taste of sorgo molasses. However, aconitic acid, in the quantities found in sorgo, will prevent the formation of crystalline sugar. The acid can be removed from the juice by the addition of lime and calcium chloride, producing calcium aconitate which can be removed by centrifugation. However, a surplus of calcium chloride will prevent the crystallization of sucrose. A rapid quantitative method was therefore needed for the determination of aconitic acid, to enhance crystallization of sucrose from sorgo.

Several methods for the determination of aconitic acid have been reported, most of which are time-consuming and plagued with interferences. Mader and Webster (1954) described a system for measuring the organic acids of sirup by partition chromatography. Ambler and Roberts (1947a, b, c) produced a decarboxylation method using potassium acetate-acetic acid solution; many organic and inorganic substances interfere. A quantitative extraction with ethyl ether, used by McCalip and Seibert (1941), is too time-consuming to be practical.

Regna and Brains (1956) described a method for the extraction of aconitic acid from molasses by using 2-butanone. They reported 93% recovery of the acid by using six extraction cycles in a three-stage unit. Final analysis was by the decarboxylation method of Ambler and Roberts (1947a). Taylor (1919) developed a qualitative test for aconitic acid by adding acetic anhydride and heating. The red color formed was unstable, finally turning brown.

This paper describes a method for extraction of aconitic acid from sorgo juice and a rapid method for measurement of the concentration.

MATERIALS

Samples of sorgo juices were obtained from Otto Coleman, Sugar Crops Field Station, U.S.D.A., Meridian, Miss. The varieties received were Rio, Wiley, Hodo, Sart, Brandes, and Mn 1056.

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Reagents used were acetic anhydride (99 to 100%), Eastman Organic Chemicals; 2-butanone, Matheson, Coleman and Bell; pyridine, J. T. Baker, reagent grade; and *cis*- and *trans*-aconitic acid, reagent grade, K & K Laboratories, Plainview, N. Y.

METHOD

Separation of Aconitic Acid from Sorgo Juice. Approximately 100 ml. of juice was centrifuged to separate the starch. The starch-free juice was shaken with Celite filter aid and filtered with suction. The clarified juice, which has a pH of 5.0 to 5.5, was lowered to pH 1.3 with sulfuric acid solution to furnish cations to convert aconitates to aconitic acid. Ten milliliters of the juice was shaken 4 minutes with 20 ml. of 2-butanone in a centrifuge tube and centrifuged 1 minute to separate the layers.

Color Development. An aliquot of the 2-butanone layer (0.1 or 0.2 ml. is usually sufficient for sorgo juices) was evaporated to dryness using a steam bath and a stream of air. Ten milliliters of acetic anhydride and 0.01 ml. of pyridine were added. The sample was mixed and after 45 minutes color was read on a Coleman Model 14 spectrophotometer at 550 mμ.

Standard Aconitic Acid Curve. Solutions of *trans*-aconitic acid were prepared in acetic anhydride to produce final concentrations ranging from 2 to 20 μg. per ml. in a volume of 10 ml. Pyridine (0.01 ml.) was added and the solution mixed and read after 45 minutes on the spectrophotometer at 550 mμ. Concentration *vs.* absorbance was plotted to give a straight line, accurate between 4 and 20 μg. per ml.

RESULTS AND DISCUSSION

Prior to the extraction of aconitic acid from sorgo juice, separate water solutions of aconitic, tartaric, citric, and l-malic acids were extracted with 2-butanone. The 2-butanone layer was evaporated to dryness and titrated with NaOH, using phenol red indicator. One extraction with 2-butanone removed 80% of the aconitic, 28% of the l-malic, 23% of the citric, and 16% of the tartaric acid. This extraction was repeated using the described colorimetric method for quantitation. Tartaric and l-malic acid gave clear solutions with acetic anhydride and pyridine, while citric acid gave a light pink color only when the concentration was at least ten times the level of that found

in sorgo juice. The only compound found which seems to interfere with the color development is water.

When the pyridine is added to the acetic anhydride containing aconitic acid, the solution turns yellow. After 5 minutes the color becomes red, changing to purple after 30 minutes. Ninety minutes after the pyridine is added, the absorbance slowly decreases.

The extraction of aconitic acid from a 20% sucrose solution gave an 84% recovery. Higher concentrations of sucrose did not increase extractability. The recovery of added *trans*-aconitic acid from Wiley sorgo juice with a single 2-butanone extraction is shown in Table I. Average recovery was 99%, more than the amount recovered from water or water-sucrose solutions. A possible explanation is that the soluble constituents in the juice in addition to sugars cause the partition coefficient to favor the 2-butanone layer to a greater degree. Mader and Webster (1954) reported that aconitic, tartaric, citric, and l-malic acids account for 94% of the organic acids in sorgo juice. Of these four, only aconitic will produce a color at the concentrations found in sorgo juice.

The ratio of the volume of 2-butanone to the volume of juice was varied, with little difference in the amount of aconitic acid extracted. A 2 to 1 ratio of 2-butanone to juice was chosen because of convenience and because their mutual solubility gave lesser phase volume change.

If the pH of the juice is not lowered, no aconitic acid is extracted from the varieties tested by this procedure. This indicates the presence of soluble sodium and potassium aconitates, with no free aconitic acid in the juice. This is contrary to the report of Ventre *et al.* (1946), who report the presence of free aconitic acid.

The aconitic acid contents of Sart, Rio, Mn 1056, Brandes, Hodo, and Wiley varieties were 3.1, 7.2, 2.5, 4.5, 2.0, and 3.1 mg. per ml. of juice, respectively.

No attempt was made to determine whether the acid was *cis*- or *trans*-aconitic. Both acids develop similar colors in acetic anhydride and pyridine; therefore, the results here show total aconitic acid. Figure 1 shows the visible spectra for *cis*- and *trans*-aconitic acid and a spectrum for the color developed in a sample of sorgo juice. Of the two aconitate isomers, Stout *et al.* (1967) report that *cis*-aconitate was low in all species or range forages, whereas the *trans*-aconitate level was as high as 6% (dry basis) in some forages.

Ventre *et al.* (1944) patented a method for separation of calcium aconitate as a by-product in the manufacture of sugar from sorgo. Later they (1946) removed 82% of the aconitic acid from sorgo juice by the addition of lime and calcium chloride. The removal of this amount should be sufficient to allow sugar to crystallize. By use of the acetic anhydride–pyridine rapid colorimetric method, it is now possible to ascertain the proper quantity of lime and calcium chloride necessary for the removal of aconitates from sorgo. This would allow sucrose to crystallize without interference of aconitic acid and excess calcium chloride.

Table I. Recovery of Aconitic Acid from Wiley Sorgo

Aconitic Acid, Mg./Ml. Juice		Recovery,
Added	Found	%
0	3.13	
1.0	4.10	97
2.0	5.08	98
3.0	6.09	99
4.0	7.01	97
5.0	8.15	100
6.0	9.12	100
8.0	11.40	103
10.0	13.05	99
12.0	15.10	100

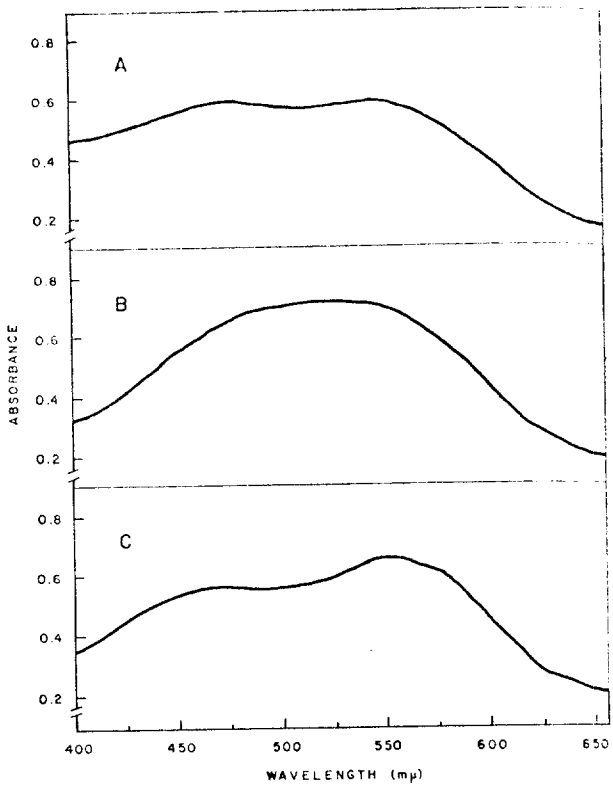

Figure 1. Visible spectra

A. Aconitic acid extracted from sorgo juice
B. *cis*-Aconitic acid
C. *trans*-Aconitic acid

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The Toxicity of Equisetum

WILLIAM F. RAPP, JR.

Recently the American Fern Journal published two short notes dealing with the toxic effect of *Equisetum* on horses. The first, by Kane (1949: 59) was an excerpt from a letter which had been published in *The New York Times*. This paper suggested that the high silica content of *Equisetum* caused "scours" in horses. The second, by Gasser (1949: 123) called attention to the fact that *Equisetum* contained a toxic alkaloid and suggested that this was the causal agent for the poisonous effect.

The poisonous nature of the various species of *Equisetum* has been known for a long time, at least to European workers. Frohner (1927: 342) gives an excellent review of the status of *Equisetum* poisoning in Europe.

The first accounts of *Equisetum* poisoning in the United States were made by Dr. F. A. Rich, a veterinarian at the Vermont Agricultural Experimental Station. In 1902 Rich published two papers (1902 and 1902b) which are identical except that they appeared in different journals. Rich and Jones (1902) also published an experimental station bulletin on the same subject, in which they stated: "This plant, *Equisetum arvense*, causes much and frequently fatal poisoning of horses throughout Vermont." Rich (in Rich and Jones 1902: 188) states: "During the past two years one of the authors in his professional work about Burlington [Vermont] has had twenty-three cases unquestionably due to this form of poisoning, while his records show forty-one cases which he has attended within five years." As a result of the amount of *Equisetum* poisoning among the horses of Vermont, Rich undertook a series of experiments in which he fed horses on *Equisetum*. As a result of these experiments, Rich (1902a: 947) described the symptoms: "Loss of coordination of muscular movement, beginning as a slight unsteadiness or uncertainty of gait, most marked when excited." On page 949, Rich states: "The horse finally dies from exhaustion induced by trauma and frequent attempts to rise."

Muenscher (1939: 27) gives the following symptoms for *Equisetum* poisoning: "At first, unthriftness and loss of weight, followed by a loss of muscle control —sways and staggers, loses power to stand and becomes very nervous and struggles violently to get up; willing to eat, but unable to rise, dies from exhaustion." The following pathological symptoms occur at the same time: "pulse becomes slow until toward the end, when it is rapid and weak; temperature below normal until animal goes down; then some fever develops, extremities are usually cold; visible lining membranes of mouth, nose and eyes become pale."

Frohner (1927: 342) states that upon autopsy horses which die from *Equisetum* poisoning show: "Hyperaemia, oedema, dropsical effusions on the brain and spinal cord, especially on the cerebellum; in cases of longer duration, hydraemia."

Most workers add that young animals develop symptoms and succumb to the poisonous effects of *Equisetum* much more rapidly than older ones. It has also been shown that the plant is more toxic when fed dry, as in hay, than when eaten in a pasture.

The majority of published statements limit *Equisetum* poisoning to horses, but Pammel (1911: 325) states: "In cattle it produces diarrhoea and cows become poor and the milk flow ceases or is checked." Gail and Hahner (1916: 14) said there was no serious effect upon cows, but that they were uncertain as to the effect upon sheep.

Rich in his work (1902a: 953) attempted to find the toxic principle of *Equisetum*, but had little success. His chemical analysis was as follows:

Moisture (as received)	14.31%
Dry matter	85.69%
	100.00%
Crude ash	19.40%
Crude protein	10.94%
Crude fiber	21.30%
Nitrogen free extract	46.30%
Crude fat (ether extract)	2.06%
	100.00%
Nitrogen	1.75%
Phosphoric acid	0.61%
Potassium oxide	4.07%
Calcium oxide	4.01%

From the above analysis he was unable to name any particular compound as the toxic principle. In view of our modern knowledge of plant toxicity it is difficult to see how Rich could hope to determine the toxic ingredient by his method of analysis.

In spite of the toxic effect on horses, *Equisetum* has been used as a drug. Young (1886: 419) studied *Equisetum hyemale* to determine its value as a medical plant. According to him, *Equisetum hyemale* was to some extent employed to remedy dropsical affections and was known to be prescribed in infusion together with digitalis and potassium acetate. As a result of his analysis, Young concluded that the effect of the medicine would have been the same if the *Equisetum* had been omitted. Young's analysis was as follows: Ash 18.2% (mostly silica); petroleum benzine exhausted from the powder 1.4% of a brownish-green semi-liquid fixed oil, which was readily saponified and was soluble in ether, chloroform and carbondisulfide; ether now took up 5.33% of a green semi-solid resin, soluble in chloroform, benzol and absolute alcohol and imparting to water or acidulated water a greenish color; 2.25% was shown by Fehling's solution to be sugar. As a result of this analysis, Young was unable to name any compound as toxic or as having medicinal properties.[1]

Various workers have stated that the toxic principle in *Equisetum* is an organic acid which has been assigned the name "equisetic acid." However, although the name "equisetic acid" occasionally appears in the literature, all efforts to trace the origin of the name have been fruitless. *The Merck Index* (1952: 390) states that equisetic acid is synonymous with aconitic acid

[1] It is interesting to note that in spite of Young's work in 1886, the twelfth edition of the U. S. Dispensatory (1918) still listed *Equisetum* as having medicinal properties, saying that it was used sometimes in dropsical and renal diseases.

(1,2,3-propenetricarboxylic acid) and on page 16 adds that aconitic acid is found in the leaves and tubers of *Aconitum napellus* L. and various species of *Achillea* and *Equisetum*. Muenscher (1939: 77), in his work on poisonous plants, states that all these plants are toxic to horses and sheep. Thus, it may be assumed that aconitic acid is one of the toxic principles in *Equisetum* poisoning.

Other workers have been interested in the alkaloids of *Equisetum*. Lomann (1904: 69) isolated an alkaloid from *Equisetum palustre* which he called "equisetin." In his recent work on plant alkaloids, Henry (1949) does not list the alkaloid "equisetin," but states that the alkaloid "palustrine" has been isolated from *E. palustre*. The alkaloid "palustrine" was isolated by Karrer and Engster (1948), but only its chemical properties were studied. Manske (1942) was able to isolate a volatile base alkaloid from *E. arvense*. He identified this compound as 3-methoxy-pyridine and stated: "There is therefore no question but that at least one species of *Equisetum* contains this, perhaps the simplest of natural alkaloids." Manske and Marion (1942) were able to isolate nicotine from *E. arvense*. Ofiejalski (1937: 470) isolated the alkaloid lycopodine from *Equisetum arvense* and found the toxic dose for cats ranged from 0.005 to 0.05 grams per kilogram of body weight. As a result of these recent studies on alkaloids, we must assume that probably the most toxic principles found in *Equisetum* are plant alkaloids.

PRESENT DAY STATUS OF EQUISETUM POISONING

Many state agricultural experiment stations publish bulletins of poisonous plants and many of these have been examined. Of the ones examined, three mentioned *Equisetum*. Stevens (1933: 17) writing on North Dakota says that some cases have been reported. Tehon, Morrill and Graham (1946: 25–27) discuss the plant in Illinois,

but do not give any details as to the frequency of poisoning. Lee and Doyle (1949: 13) list the plant as of minor importance in Indiana.

In addition to examining bulletins on weeds, questionnaires were sent to a number of experiment stations. In Massachusetts, Dr. K. L. Bullis (letter, Jan. 23, 1951) stated that there seemed to be no record of cases of *Equisetum* poisoning. Dr. W. W. Robbins (letter, Feb. 7, 1951) reported that in California there are some losses of livestock due to *Equisetum*, but that *Equisetum* is not regarded as one of the primary poisonous plants. Dr. John H. Helwig, of The Ohio State University (letter, Jan. 22, 1951) claimed that they have no experience with *Equisetum* poisoning in Ohio. Dr. W. D. Bolton, of the University of Vermont, stated (letter, Jan. 22, 1951) that *Equisetum* poisoning probably occurs occasionally in the state. In Iowa, Dr. Richard W. Pohl mentioned (letter, Jan. 24, 1951) that as far as he knew, no cases of *Equisetum* poisoning had been reported. In Kansas, Dr. L. M. Roderick, of Kansas State College, (letter, Jan. 20, 1951) said that their attention had never been called to any cases of *Equisetum poisoning*. In Michigan, Dr. R. A. Rummells, of Michigan State College, (letter, Jan. 19, 1951) stated that *Equisetum* poisoning occurs in Michigan, but that the cases reported are never very numerous. In Illinois, Dr. C. C. Morrill, of the University of Illinois, (letter, Jan. 19, 1951) said that they do not see cases of *Equisetum* poisoning very often.

CONCLUSIONS

It would appear that *Equisetum* poisoning in horses is due to the presence of aconitic acid and to the presence of one or more alkaloids. The distribution of *Equisetum* poisoning in North America is not well known, because with the decrease in the number of horses on American farms, marked cases of *Equisetum* poisoning are seldom seen today.

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ON THE EXCRETION OF CITRIC ACID BY RATS AFTER ORAL INTAKE OF VARIOUS ORGANIC ACIDS

by

P.J. Simola and Terttu Kosunen

(Entered on July 24, 1938)

In an earlier report, Simola, Krusius and Alapeuso reported on experiments, in which an intense increase in the citric acid values in the urine, determined by the colorimetric pentabromoacetone method, was found after the administration of pyruvic acid, α-ketoglutaric acid, succinic acid, fumaric acid, malic acid, β-hydroxybutyric acid and acetoacetic acid. In the following are reported the results of a more thoroughgoing series of experiments, in which we tested the effect of a series of aliphatic carbonic acids, which possess more or less theoretical interest with regard to the mechanism of citric acid formation.

For experimental animals in these investigations we employed full-grown rats, to which the substances to be tested were administered orally as sodium salts in molecular proportion. The amount of the substances always corresponded to 60 mg sodium per 100 g rat weight. For every experiment, two rats were usually used; the urine which they excreted in the course of 24 hours was collected. The citric acid content of this collected urine was then analyzed according to the pentabromoacetone method. The substances tested were: pyruvic acid, pyruvic acid aldol, α-ketoglutaric acid, oxalacetic acid, succinic acid, fumaric acid, maleic acid, malic acid, glutaric acid, adipic acid, malonic acid, butyric acid, isobutyric acid, β-hydroxybutyric acid, crotonic acid, citraconic acid, aconitic acid, glycolic acid and gluconic acid.

The results of the experiments are shown in the appended table, in which the content of the citric acid determined by means of the pentabromoacetone method is given both in mg per animal for 24 hours and in mg% of the total urine.

As is revealed by the table, the amounts of citric acid excreted during 24 hours are higher after all the administered acids than in the control experiment, where only sodium carbonate was administered. The amount of citric acid excreted during the 24 hours was highest after the administration of pyruvic acid aldol and glutaric acid, lowest after gluconic acid and adipic acid. When the acids are arranged in decreasing order according to strength of effect, the following is found: pyruvic acid aldol, glutaric acid, malonic acid, succinic acid, fumaric acid, aconitic

acid, citraconic acid, crotonic acid, malic acid, butyric acid, glycolic acid, α-ketoglutaric acid, β-hydroxybutyric acid, maleic acid, pyruvic acid, isobutyric acid, oxalacetic acid, gluconic acid and adipic acid.

	A. Verabreichte Substanz	B. Ausgeschiedene Citronensäure pro 24 St. in mg	C. Citronensäuregehalt des Sammelharnes in mg %
1.	Ohne jede Verabreichung	0,8	16
2.	Natriumcarbonat	2,9	44
3.	Brenztraubensäure	11,6	290
4.	Brenztraubensäurealdol	57,0	950
5.	α-Ketoglutarsäure	14,6	366
6.	Bernsteinsäure	35,5	418
7.	Fumarsäure	31,4	275
8.	Maleinsäure	13,7	210
9.	Äpfelsäure	17,3	287
10.	Oxalessigsäure	8,6	216
11.	Normalbuttersäure	15,8	158
12.	Isobuttersäure	9,8	109
13.	β-Oxybuttersäure	14,6	162
14.	Crotonsäure	18,6	133
15.	Malonsäure	37,3	583
16.	Glutarsäure	51 8	1150
17.	Adipinsäure	5,0	200
18.	Citraconsäure	31,9	638
19.	Aconitsäure	32,3	413
20.	Glykolsäure	15,0	158
21.	Gluconsäure	7,9	71

Key:
A.= administered substance B.= citric acid excreted in 24 h in mg.
C.= citric acid content of the total urine in mg%.

1.= without any administration
2.= sodium carbonate
3.= pyruvic acid 4.= pyruvic acid aldol 5.= α-ketoglutaric acid
6.= succinic acid 7.= fumaric acid 8.= maleic acid
9.= malic acid 10.= oxalacetic acid 11.= butyric acid
12.= isobutyric acid 13.= β-hydroxybutyric acid
14.= crotonic acid 15.= malonic acid 16.= glutaric acid
17.= adipic acid 18.= citraconic acid 19.= aconitic acid
20.= glycolic acid 21.= gluconic acid

The order is different when the acids are arranged according to the percentage content of the citric acid in the total urine of 24 hours: glutaric acid, pyruvic acid aldol, citraconic acid, malonic acid, succinic acid, aconitic acid, α-ketoglutaric acid, pyruvic acid, malic acid, fumaric acid, oxalacetic acid, maleic acid, adipic acid, β-hydroxybutyric acid, butyric acid, glycolic acid, crotonic acid, isobutyric acid and gluconic acid.

Thus in both series the effect of pyruvic acid aldol and glutaric acid is strongest, that of gluconic acid weakest. It is moreover remarkable that oxalacetic acid, which causes an intense increase in the citric acid values in the kidneys and testes, as was found in in vivo experiments in this laboratory, shows a re-

latively weak effect. The substances tested behaved differently in other respects as well in the in vivo experiments from the way they behaved in the in vitro experiments, which were carried out simultaneously with our investigations, and which will be reported elsewhere by Hallsten and Linola.

Experimentation with rats and the judging of the results is made difficult moreover by the fact that the amount of urine excreted in 24 hours by rats undergoes considerable variations. (In our administration experiments we found variations of from 2 to 14 ccm.) Furthermore, the substances are absorbed from the intestinal tract in various ways. Experiments which will hopefully shed more light on the question are at present in progress; the above-reported experiments are being repeated simultaneously, using different amounts of the substances. At the same time, a series of new substances is being tested, and animals larger than rats are being used as experimental animals. For the purpose of control of the values obtained with the pentabromoacetone method, the enzymatic citric acid determination method of Thunberg will also be used.

Finally, it should be mentioned in this connection that in these experiments, besides citric acid formation, keto acid excretion was also considered. By means of the usual 2,4 - dinitrophenylhydrazine reagent it was determined that keto acid excretion was clearly increased after the administration of all the substances named, with the exception of butyric acid, isobutyric acid, and glycolic acid. The effect of pyruvic acid aldol and glutaric acid seemed to be the strongest. The nature of the excreted keto acids was not examined more closely in all cases. In a portion of the cases, there is a definite increase in the excretion of α-ketoglutaric acid.

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TRANS-ACONITIC ACID AND THE MAGNESIUM STATUS OF GUINEA PIGS AND SHEEP

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ABSTRACT

Single oral doses of sodium *trans*-aconitate slightly reduced the blood serum Mg levels of guinea pigs, but not of sheep. Feeding guinea pigs on a diet containing 6.8% *trans*-aconitate had little effect on growth or on serum Mg levels. *Trans*-aconitate reduced the oxidation of citric acid-1,5-^{14}C by liver homogenates, but did not reduce the *in vivo* oxidation of glucose-U-^{14}C or citric acid-1,5-^{14}C to carbon dioxide. It is concluded that *trans*-aconitate is not a major factor in determining the Mg concentration in serum, nor does it have a toxic effect *in vivo*.

INTRODUCTION

Burau and Stout (1965) found high concentrations of *trans*-aconitic acid in several range grasses in California during early spring and discussed the possibility that this acid may be partially responsible for hypomagnesaemia. A role for tricarboxylic acids in influencing the level of serum magnesium in calves has been reported by Burt and Thomas (1961) who showed that feeding sodium citrate at the level of 1% of the diet reduced serum magnesium from 2.11 to 1.86 mg/100 ml.

Burau and Stout (1965) point out that there may also be biochemical consequences to animals ingesting large amounts of *trans*-aconitic acid, since this acid is known to inhibit the citric acid cycle enzyme aconitase (Saffran and Prado 1949). However, since this inhibition was only demonstrated *in vitro*, it seemed desirable that the action be tested *in vivo*.

This paper reports the results of experiments on the effect of sodium *trans*-aconitate on serum magnesium levels and on the oxidation of radioactive citric acid or glucose to carbon dioxide.

EXPERIMENTAL

Preparation of sodium trans-*aconitate*

Trans-aconitic acid was prepared by dehydrating citric acid with concentrated H_2SO_4 (Blatt 1944). The identity of the acid was confirmed by infra-red spectroscopy, melting-point (195–200°C), paper

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chromatography, and high-voltage electrophoresis (Gross 1956). The acid was converted to the sodium salt by neutralising an aqueous solution of the acid to pH 8 with 3 N NaOH.

Experiment 1: Effect of sodium trans-*aconitate on magnesium levels in guinea pig serum*

Male guinea pigs weighing between 360–490 g were divided into two groups of seven with comparable serum Mg levels. They were fed on lucerne meal nuts containing 0.19% Mg on dry weight basis and on fresh grass. The sodium salt of *trans*-aconitic acid was dosed orally to one group at the rate of 3.4 mM/kg body weight, which corresponded to a level of about 1.3% of acid in the dietary dry matter. Blood samples were obtained by cardiac puncture, at 2–3 hr before dosing, and 1–2 hr and 21 hr after dosing. The animals had access to water, but food was withdrawn from 12 hr before dosing to 4 hr after dosing. At the end of one week the treated and control groups were interchanged and the experiment repeated.

Experiment 2: Effect of feeding a high level of dietary sodium trans-*aconitate to guinea pigs*

Three male guinea pigs were fed on the above diet to which was added 6.8% of sodium *trans*-aconitate. The animals were compared with a control group of three animals for weight gain and general health; and on slaughter (after 13 days) for serum Mg concentrations. The diet of the control group contained added $NaHCO_3$, so the Na content of the feed was the same for both groups.

Experiment 3: Effect of a single massive dose of trans-*aconitate on serum magnesium levels in sheep*

Six hoggets weighing between 20–28 kg were fed on chaffed meadow hay (Mg = 0.28% of dry weight). Blood samples were withdrawn through polythene or teflon catheters inserted into the jugular vein, at intervals of 4 hr between 9 a.m. and 1 a.m. daily. After the first 5 p.m. blood sampling, 3 sheep were dosed by stomach tube with 0.29 M of sodium *trans*-aconitate, equivalent to 10% of the dietary dry matter. The 3 control sheep received 0.87 M of $NaHCO_3$ to provide an equivalence of sodium ions. After 48 hr the treatment of the two groups was reversed.

Experiment 4: Formation of labelled CO_2 from sodium citrate-1,5-^{14}C

Guinea pig liver homogenates prepared in a KCl-$KHCO_3$ solution (Umbreit, Burris, and Stauffer 1964) were incubated at 38°C in Warburg flasks containing labelled substrate. After the homogenates had been killed with 10 N H_2SO_4, CO_2 was trapped on filter papers soaked in 20% (w/v) KOH.

Guinea pigs were injected intra-peritoneally with labelled substrate and placed inside a metabolic cage through which CO_2-free air was pumped. The respired gas was dried by passing through concentrated H_2SO_4 and anhydrone (magnesium perchlorate) and the CO_2 trapped in carbonate-free 3 N NaOH contained in weighed bubblers.

The carbonate in solution was precipitated with $BaCl_2$ and the $BaCO_3$ collected on filter paper discs. Radioactivity in the $BaCO_3$ was measured in a windowless gas-flow counter. The counts, corrected for background radiation and for self-absorption to infinite thinness, are expressed as counts per minute.

Estimation of Mg

Serum was diluted 100 × with 1% (w/v) HCl containing 1% (w/v) Sr and analysed for Mg by atomic absorption spectroscopy (Willis 1960).

RESULTS

Experiment 1

The effect of dosing *trans*-aconitate on the Mg levels in guinea pig serum is shown in Table 1. The results show that, after correcting for the variation in pre-dosing levels by covariance analysis, the serum Mg was slightly lower in the treated group than in the control after 2 hr. After 21 hr no difference was found between the two groups.

Experiment 2

Guinea pigs fed a diet containing 6.8% of *trans*-aconitate did not show any ill effects after 13 days of feeding, their weight gain and appearance being similar to those of the controls. Over the full experimental period of 13 days the mean weight gain for the dosed group was 28 g compared with 34 g for the control animals. In the dosed group there was a weight loss in the first 2 days, possibly because the high *trans*-aconitate content of the diet was unpalatable. Over the last 11 days the weight gain for the dosed animals averaged 44 g compared with 34 g for the undosed animals. The serum Mg levels were similar in both groups.

TABLE 1—Effect of Dosing Guinea Pigs with 3.4 mM of Sodium *trans*-aconitate/kg Body Weight on Serum Magnesium Levels

Time after Dosing (hr)	Control	Treated	Control less Treated	
	mg Mg/100 ml			Adjusted(1)
0	3.01	2.96	+0.05±0.154	—
2	2.64	2.31	+0.33±0.161	+0.32±0.109(2)
21	2.91	2.83	+0.08±0.197	+0.03±0.113

(1) adjusted by covariance analysis for pre-dosing level.
(2) $p<0.05$.

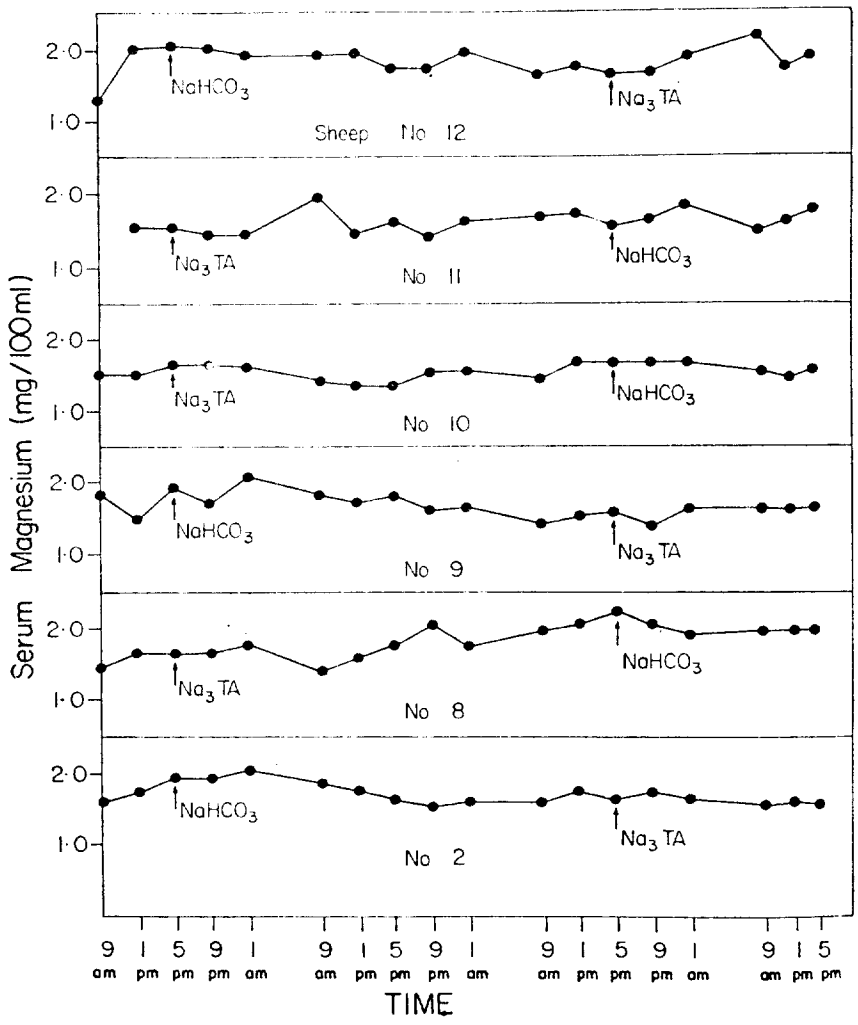

Fig. 1—Effect on serum magnesium of oral doses of sodium *trans*-aconitate or sodium bicarbonate.

Experiment 3

The results of dosing the neutralised acid to sheep are shown in Fig. 1. No differences were found in the serum Mg levels at any of the sampling periods.

Experiment 4

The *in vitro* toxic action of *trans*-aconitate on citric acid cycle activity was demonstrated by adding sodium citrate-1,5-^{14}C (0.013μM) to a guinea pig liver homogenate in the presence or absence of *trans*-aconitate. The total counts added to 2.0 ml of homogenate were 248,000 c.p.m. After 30 min at 38°C, the radioactivity in the CO_2 from the control sample was 100,000 c.p.m. compared with 41,000 c.p.m. and 16,000 c.p.m. respectively when 5.8 mg or 17.4 mg of *trans*-aconitate were added. These values indicate 59% and 84% inhibition of aconitase.

No reduction in the formation of labelled CO_2 was found in animals injected intra-peritoneally with radioactive citric acid or glucose before or after dosing with *trans*-aconitate. The results are shown in Table 2.

TABLE 2—The Effect of *trans*-aconitate on the *in vivo* Oxidation of Citric Acid-1,5-^{14}C or Glucose-U-^{14}C to Radioactive CO_2

trans-aconitate Dosing		Labelled Substrate[1] (1μc)	Radioactivity in CO_2	
Amount (mg/g body wt)	Time before Substrate Injection (min)		Pre-dosing	Post-dosing
0.56	30	Glucose-U-^{14}C	266,000 285,000	290,000
0.35	240, 180, 120, and 60	Glucose-U-^{14}C	300,000 290,000	280,000
0.56	30 and 5	Citric acid-1,5-^{14}C	284,000 308,000	302,000
0.56	30 and 5	Citric acid-1,5-^{14}C	302,000 300,000	293,000

[1] Labelled substrates (1 μc in 0.2 ml of sterile 0.9% NaCl solution) injected intra-peritoneally.
[2] Radioactivity expressed as counts/min in CO_2 collected for 3 hr post-injection. Two values for the pre-dosing period were collected on consecutive days.

DISCUSSION

Though single large doses of *trans*-aconitate slightly lowered the serum Mg of guinea pigs, single doses amounting to about 10% of the daily dry matter intake did not reduce the serum Mg levels of sheep. Unlike the study reported by Burt and Thomas (1961) on the effect of dietary citrate on serum Mg levels in calves, where a daily intake of citrate amounting to 1% of the diet was fed over 8 weeks, this experiment involved only one massive dose. Prolonged dosing may be more effective in lowering serum Mg, but this was not observed in guinea pigs fed on large amounts of *trans*-aconitate for 13 days. It is concluded from these experiments that *trans*-aconitate acid does not play a major role in controlling serum Mg levels. However, under conditions of marginal Mg status, this acid might be a minor factor leading to hypomagnesaemia.

Likewise, under the experimental conditions used in these studies, *trans*-aconitate does not have any *in vivo* toxic effect, since no effects on growth rate of guinea pigs or on citric acid cycle oxidations were found. As the previously reported toxic action of *trans*-aconitate could be demonstrated using liver homogenates, the explanation for the lack of toxicity *in vivo* may be the impermeability of intact liver cells to this tricarboxylic acid.

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Miss R. Sawyer and Miss P. Gunn: technical assistance; Mr F. S. Pickering: Mg analyses; and the U.S. Public Health Service: grant (5 R05 TW 00315-02).

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